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THE BINDING OF 5 HYDROXYTRYPTAMINE TO PLATELETS AS APPLIED TO THE STUDY OF PLATELET DISORDERS

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "THE BINDING OF 5 HYDROXYTRYPTAMINE TO PLATELETS AS APPLIED TO THE STUDY OF PLATELET DISORDERS", submitted by Marilyn B. Leslie in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

When carefully isolated from fresh blood, mammalian platelets contain 5 hydroxytryptamine (5HT or serotonin). Further, platelet 5HT concentration can be increased manyfold when platelets are incubated at 37°C in the presence of the amine. This process is one of active transport as it can operate against large concentration gradients. 5HT appears to be metabolically inert within the cell (1,2).

The initial investigation was of platelet survival in humans, using 5HT as a platelet "tag". Platelet 5HT levels in human volunteers were elevated by oral administration of at least 160 mg of 5HT per day. Following this, the decline in 5HT level was determined by daily assay, and the half-life (T1/2) calculated. The T1/2 for the platelet 5HT tag in three normal individuals was found to be 3.5 to 5 days.

This method of determining platelet half-life proved unreliable when it was found that normal persons varied widely in their ability to increase platelet 5HT even after prolonged oral administration of 5HT. Further, a thrombocytopenic patient on whom there was considerable indirect evidence indicating the existence of platelet antibodies, failed to exhibit any increase in an exceedingly low platelet 5HT value. As a primary application of platelet half-life estimation would be the evaluation of thrombocytopenia, the investigation was concluded.

However, if, as it appeared, platelet antibodies did act to inhibit the binding of 5HT by platelets, a much needed *in vitro* method for the detection of platelet antibodies could be developed.

To this end, rabbits were immunized with human platelets to provide a source of anti-human-platelet serum. This serum as well as that of normal volunteers and patients was tested by incubation with pooled normal human platelets and 5HT-C^{14} in the presence of complement (C'). With each series of tests, an antibody-free saline control was run to establish "100%" or the maximum possible 5HT binding by the platelets. Sera which allowed over 80% uptake as compared to the control were considered to be free of platelet antibodies (negative). Sera causing an uptake less than 80% of the control were considered to contain platelet antibodies and were graded 1+ to 4+ depending upon the dimension of difference from the control. That platelet antibody was the cause of diminished uptake of 5HT by platelets was somewhat confirmed by the fact that absorption with human platelets removed the inhibitory substance.

Interpretation of results on patients' sera were complicated by the fact that there was not any suitable alternate method by which to document the presence of platelet antibodies. However, sera of patients who had multiple transfusions and who therefore were likely to have developed platelet antibodies gave results similar to those of the sensitized rabbit serum. The inhibition of uptake of 5HT by platelets indeed appears to be a promising procedure for the detection of platelet antibodies.

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I. INTRODUCTION

Thrombocytopenia is a pathological condition in which there is a decrease in the number of blood platelets. If the platelet number, normally 150,000-350,000/mm^3 blood, falls to 5,000-10,000/mm^3, there may be hemorrhage from mucous membranes and bleeding into the skin, resulting in the formation of purple patches (petechiae). The condition may be chronic or acute. The frequently used term "Idiopathic Thrombocytopenic Purpura" (ITP) reflects the fact that in many instances little is known of the etiology of the condition.

The onset of the disease is sometimes associated with blood transfusions, the administration of drugs, or viral infections. Low platelet levels also occur in the newborn, as a result of maternal sensitization to foetal platelet antigens, and in young children where thrombocytopenia not infrequently undergoes spontaneous remission.

Investigations by Gardener (3) and others have regularly shown a shortened platelet life-span in ITP. Baldini (4) is of the opinion that in many, if not all instances, the increased platelet destruction is an immunological process.

Investigations relevant to this theory have been severely hampered by the lack of simple and reliable <u>in vitro</u> methods of platelet antibody detection. Methods available, reviewed by Colombani (5), involve platelet agglutination as a

result of reaction with antibodies, complement (C') fixation by the antigen-antibody reaction, and estimation of platelet lysis by platelet antibodies in the presence of C'.

However, *in vivo* methods for the documentation of the effect of platelet antibodies have become established. These are essentially platelet survival or half-life tests, which involve tagging isologous or autologous platelets, infusing them into the patient and taking blood samples periodically to establish the rate of loss of the tag from the circulating platelets. Cr^{51} is perhaps the most commonly used tag.

Melmon (6) noted that when 5 hydroxytryptamine (5HT or serotonin) is administered orally, it is capable of being strongly bound by platelets in the circulation. It was possible that after accomplishing a maximal platelet 5HT level, daily assays of platelet 5HT would reflect the loss of the original platelet population from the circulation. The possibility of tagging platelets *in vivo* with a physiological non-radioactive substance constituted a decided advantage over other methods. It was decided to further investigate this method of determining platelet survival.

Results of initial investigations and work by Bridges *et al*. (7) led to the major phase of this work. It appeared that the presence of platelet antibodies inhibited binding of 5HT by normal platelets *in vitro*. If true, a simple *in vitro* test could be devised for platelet antibody detection. Such a test could not only assist the diagnosis of thrombocytopenia, but the selection of compatible platelets for transfusion.

II. PLATELETS AND 5 HYDROXYTRYPTAMINE

A. The Blood Platelet

1. Introduction

Platelets are granulated fragments of megakaryocytes which circulate in the blood stream; their function is hemostasis, and their average life span is 7-12 days.

Platelets were first described as fatty particles of chyle. It later became established that they were cellular particles and after various suggestions as to their origin, it was established in 1910 that they were produced from the megakaryocytes. The bone marrow is thought to be the primary site of production (8).

In vitro study of cultures of megakaryocytes have shown that numerous pseudopods are formed from cytoplasm and that the platelets eventually bud off at the extremities.

Normal platelets vary from 2-4 μ in diameter and usually 7-8 μ^3 volume. The electron microscope can be used to distinguish the various types of platelets which adhere to a glass slide. Nearly all of the platelets are in the round form if the slide is fixed immediately after exposure. When fixation is delayed a few minutes, protoplasmic processes develop (dendritic forms) and finally these processes become joined by cytoplasmic veils, resulting in the spread form.

A nucleus has never been demonstrated in the platelet (8). Nevertheless, the cell is extremely active metabolically.

Glycolysis is the main source of energy and at least 30 enzymes are known to be present (9).

Sulfhydryl (SH) dependent enzyme systems appear to be essential to the survival of platelets. Addition of "SH inhibitors" to platelet suspensions result in the disappearance of many platelets (10).

In the cytoplasm are granules which under some circumstances become clumped in the center of the platelet, taking on the appearance of a nucleus. It is probable that at least some of these granules are lysosomes since such enzymes as acid phosphatase and beta-glucuronidase have been identified (10). The granules also contain high levels of ATP and 5HT (11).

2. Function of Platelets

The tendency of an individual to bleed is obvious if the platelet number falls below 50,000/mm^3. Conversely, the main function of platelets is hemostasis. This function depends upon those properties enabling (a) capillary endothelial support; (b) production of hemostatic plugs subsequent to initiation of ATP degradation and the aggregation of platelets; (c) the ability to release a lipoprotein material, Platelet Factor 3, which may initiate the coagulation mechanism (10).

However, the severity of bleeding as well as the tendency to bleed in thrombocytopenia varies not only between individuals but from one time to another within one individual. Although low platelet counts hinder hemostasis, bleeding may start and subside spontaneously in patients while there is no significant change in a low platelet count. Further purpuric

bleeding frequently appears at higher platelet levels when thrombocytopenia develops rapidly than when the process is chronic.

3. Platelet Antibodies

For simplicity, immunization processes which can lead to thrombocytopenia will be categorized as follows: (i) isoimmunization, which may ultimately cause thrombocytopenic purpura in the newborn, or in patients after having ABO and Rh compatible blood transfusions; (ii) allergic immunization. The latter, which may lead to the destruction of autologous platelets, may be a mechanism in some patients currently diagnosed as Ideopathic Thrombocytopenic Purpura.

(i) Isoimmunization

Purpura of the newborn, for example, has only recently been demonstrated to be of immunologic nature. The incidence is low (1 case in 5,000 to 10,000 live births), but may occur as early as the first pregnancy. Its mechanism is the same as that of hemolytic disease of the newborn. A difference in parents' blood group results in the conception of a child whose platelets bear an antigen which is absent from the mother's platelets. The leakage across the placenta allows this antigen into maternal circulation, causing the production of maternal antibodies.

Maternal 7S gamma globulin antibodies enter the fetal circulation and react with the antigen-bearing fetal platelets to produce a thrombocytopenia which lasts until the antibody is eliminated. The serologic diagnosis is made by the detection

in the mother's serum of an antibody which is active against antigens of the father's and child's platelets (5).

Similarly, five cases of post-transfusion purpura, described by Comumbani (5), involved women with histories including at least one pregnancy or previous transfusion. These individuals developed thrombocytopenia 5-7 days after transfusion. Three to four weeks later, total recovery occurred either spontaneously or following corticosteroid therapy, and the platelet count returned to normal levels. It is of interest that when the purpura occurred, antibodies detected in the serum were inactive against the individual's own platelets, but active against donor platelets.

Shulman (12) and Nachman (14) have hypothesized that in such cases, the transfusion of ABO-Rh compatible blood, or pregnancy initiates the production of an isologous platelet antibody. On further transfusion, donor platelets are sensitized by this antibody and eliminated from the circulation of the recipient. The subsequent destruction of autologous platelets is a result of the passive absorption to the platelet surface of an antigen-antibody complex which is released during the destruction of transfused platelets. Spontaneous remission may begin only when supply of the complex has been exhausted.

(ii) Allergic Immunization

Purpura has also been known to develop 8-10 days after a sensitizing dose of a drug or chemical. Levine (13) established that, in order for a drug to induce an immune response, the drug or perhaps an in vivo degradation product of the drug must be able to bind strongly to tissue protein. He hypothesizes

that antibodies synthesized in response to such a conjugated protein antigen will show structural specificity toward the haptenic group (formed by the reaction of the drug and protein), toward the amino acid residue through which the hapten is bound to protein, and possibly also toward structural configurations of the autologous carrier, which may be a platelet protein.

If quinidine, frequently found to be the cause of drug sensitive thrombocytopenia, is administered orally to normal individuals, and plasma from patients susceptible to quinidine induced purpura is then infused, the destruction of normal autologous platelets will begin immediately. Spontaneous remission will occur some days later. Levine's hypothesis of drug (hapten) absorption to platelets could be a suitable explanation of these events (above). However, the preferred theory is that the drug alone or in combination with a non-platelet protein constitutes the antigen, which in combination with the antibody absorbs non-specifically onto the platelet causing its destruction (30).

B. 5 Hydroxytryptamine (5HT)

5HT was discovered as a normally occurring amine, as a result of independent studies on "Thrombocytin"--the potent vasoconstrictor in serum, and on "Enteramine"--a substance found in the gastro-intestinal mucous membrane, able to stimulate smooth muscle contraction. It is established today that both are in fact 5HT.

Tryptophan is the parent amino acid of 5HT, and normally 1% of the absorbed dietary amino acid is converted to

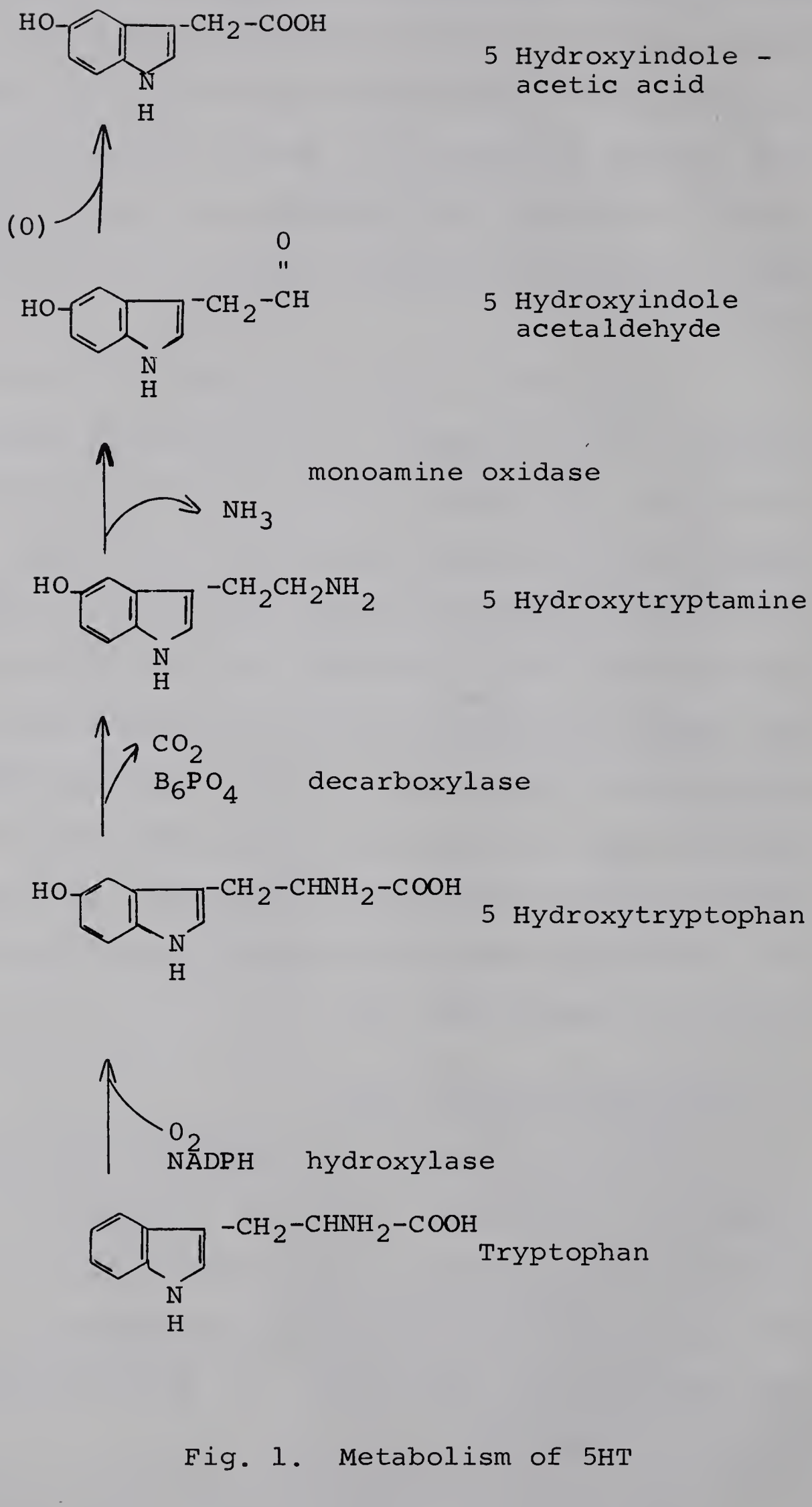

Fig. 1. Metabolism of 5HT

serotonin (2). Tryptophan is hydroxylated by an enzyme which has been shown to occur in the liver and is possibly present in other tissue. The 5 hydroxytryptophan is converted by decarboxylase to 5HT (Fig. 1).

Following its formation *in vivo*, 5HT is stored in various sites, one of which is blood platelets. Although the blood platelet may contain a considerable amount of 5HT, the lack of decarboxylase in the platelet suggests that serotonin is not manufactured but merely concentrated there. The majority of 5HT is eventually metabolized by oxidative deamination to form 5HIAA. The enzyme which catalyzes this reaction is mono amine oxidase, a stable mitochondrial enzyme that is widely distributed in tissue (2).

C. 5HT Binding by Platelets

1. Introduction

In vivo and *in vitro*, platelets are capable of binding and maintaining concentrations of 5HT many times that of their media. It is probable that the 5HT is held irreversibly unless there is much 5HT free in the medium; in which case an exchange may occur (for example, in carcinoid syndrome (15), where blood 5HT levels are markedly increased). Since no physiological purpose for the accumulation of 5HT by the platelet has been established (2), it has been considered potentially useful as a platelet tag.

The movement of 5HT across the platelet membrane is maximal at 37^{o}C, it is inhibited by the presence of cyanide, iodoacetate, and cardiac glycosides. The mechanism further appears to be related to a cation exchange system as potassium

is displaced as 5HT goes into the cell (2,9).

In 1959, Brodie (16) working with a wide range of concentrations of 5HT, demonstrated that 5HT binding by platelets *in vitro* involves two mechanisms. The first is probably responsible for binding *in vivo* and *in vitro* when the environmental concentration of 5HT is "low" (perhaps up to 50 μg/ml). This mechanism is active and rapid, inhibited by Reserpine which acts against the stimulation of glucose and ATP (18) in a 5HT-saline medium.

At higher concentrations of 5HT in the media, data indicates that 5HT accumulated by platelets is primarily a result of diffusion (11,16). Further, Reserpine, in the absence of glucose or ATP, appears to have a conservative effect on the rate of loss of accumulated cellular 5HT (18).

The subcellular localization of 5HT has been partly elucidated in the past few years. The amine has been shown to exist in the granule fraction of rabbit platelets, probably in bound form. The platelet membrane does not appear to contain 5HT, and Haverbach is of the opinion that small amounts of free 5HT sometimes reported in the cell is probably due to mechanical rupture of some of the granules(2).

2. Modes of Platelet Study

The clinician's evaluation of thrombocytopenia and the therapy he subsequently prescribes, is largely based on his personal experience. The major mode of laboratory assistance is Platelet Counts. However, these, when done with the light microscope have a large margin of error. Further, there is no ideal method of predicting the survival of platelets transfused

to control bleeding. The detection of platelet antibodies in serum is not a reliable nor a routinely available test.

An alternative method to study platelet disorders is to study platelet survival. Perhaps the most widely used method is Gardener's (3), in which platelets are tagged *in vitro* with sodium chromate (Cr^{51}), and infused into the patient. Blood samples are taken daily to document the gradual disappearance of the Cr^{51} from the circulating platelets. The slope of this decline curve is indicative of the survival of the platelets *in vivo*.

Since the test is often carried out in the presence of thrombocytopenia, isologous platelets must be transfused and the patient faces the hazards of anti-platelet antibody formation or reaction, infection, and exposure to radiation.

3. Possible Advantages of 5HT in Platelet Study

5HT has been successfully used as a tag for platelet survival studies by several authors (10,19). 5HT-C^{14} has been injected intravenously into the patient, for *in vivo* labelling of platelets. Platelets may also be tagged *in vitro*, by incubation with 5HT in plasma, and then infused into the patient. Daily blood samples are taken in either case, in the same way as the Cr^{51} method.

The advantages of 5HT center on the fact that because platelets selectively bind the amine and other blood cells do not, less manipulation is needed during the labelling procedure. However, when Melmon (6) published findings that 5HT administered orally might conceivably be an efficient platelet tag, there was yet another possible advantage, that of labelling

autologous platelets by giving the amine by mouth. Trials on seven volunteers were initiated to test this possibility.

The process of platelet binding 5HT was itself of interest with regard to the study of platelet antibodies. Bridges (7) noted that in sera where platelet antibodies were suspected to be present but often undetected, there was an anti-platelet-5HT-binding factor present. Similar observations were made in one of the above volunteers. There was strong clinical evidence that the serum contained platelet antibodies. At the same time, the platelets were found to contain very little 5HT, nor did they bind any during the period of oral administration. The resultant investigation was designed therefore to study the feasibility of developing a sensitive method for the detection of serum platelet antibodies by measuring the inhibitory effect of platelet antibodies upon the uptake of 5HT by normal platelets.

III. EXPERIMENTAL

A. General

1. Until platelets are dissolved (i.e. for counting, analysis, etc.), all surfaces contacted must be coated with silicon oil or be plastic. Otherwise, platelet viability will be reduced.

2. Platelets or platelet suspensions are kept at 4°C whenever procedures are delayed.

3. Pasteur pipets used to transfer and suspend platelets are siliconed and have fire-smoothed tips.

4. Platelets are washed by repeated gentle aspiration of the platelet button in wash liquid, in and out of a Pasteur pipet. The resulting suspension should become free of visible clumping of the cells.

5. By the blood collection method outlined below, the platelet yield from 1 ml blood is a platelet button containing approximately 0.2 mg protein.

6. An "anti-platelet-5HT-binding factor" was developed and detected in rabbit serum, and was also detected in patient serum, clinically suspected to contain platelet antibodies. This factor may, for brevity, be called an "antibody", although only indirect support for this assumption is available.

B. Blood Collection Methods

Blood for platelet 5HT assay is collected using an 18 gauge disposable needle, and a siliconized syringe. It is

transferred gently into containers with 1 ml anticoagulent (2 gm disodium EDTA and 0.7 gm NaCl per 100 ml) for each 9 ml of blood. Tubes are immediately refrigerated and are kept at 4°C until centrifugation, also at 4°C, begins.

Pooled blood to supply platelets for in vitro 5HT binding studies is collected in a 5 ml Becton Dickinson Evacuated Specimen Tube (Vacutainer), which has treated surfaces to minimize hemolysis and contains dry EDTA. A siliconed size 20 needle is attached for the venipuncture.

Sufficiently viable platelets for use in platelet 5HT binding studies, are harvested if the blood is left in the original tubes at room temperature up to 24 hrs. Pooling of the blood must be followed immediately by preparation of the platelet button. Centrifugation may be done at room temperature.

Serum to be tested for the presence of platelet antibodies is prepared from blood collected in a Vacutainer using a size 20 needle. The blood is allowed to clot for at least 4 hrs at room temperature before the serum is removed and stored at -20°C.

C. Platelet Preparation for 5HT Assay or Binding Studies

1. Separation of Platelets from Blood

The blood is first centrifuged at 1,000 r.p.m. for 10 minutes. A Pasteur pipet is used to remove the cloudy platelet rich plasma (PRP), and the top 10% of the red cell layer to a second tube. After centrifugation of this tube at 1,000 r.p.m. for 10 minutes, the PRP is taken from the settled red cells and spun at 3,000 r.p.m. for 10 minutes, the clear supernatant

decanted and the last drops blotted from the mouth of the tube. To lyse any remaining red cells, the platelets are washed in about 5 ml of 1% ammonium oxalate at room temperature. The platelets are respun at 3,000 r.p.m. for 10 minutes, the clear supernatant discarded, and the suspension washed with normal saline (kept at 5°C). After final centrifugation, the tube is inverted to drain off the clear supernatant, the last few drops again being blotted from the mouth of the tube. If the resulting platelet button is to be stored, it is left in an inverted tube, at 4°C.

2. Platelet Suspension for 5HT Binding Studies

In order to have a relatively constant concentration of platelets in the various binding trials, a method was developed in which turbidity is used to determine the volume in which a platelet button is to be suspended.

After preparation of the platelet button (above), it is initially suspended in 4.0 ml of incubation saline (Section IV.C.1.). A 4.0 ml aliquot of the saline is also put in a cuvette and used to zero a colorimeter set at 640 mμ. The optical density (OD) of a 5% solution of $CuSO_4$ is determined. Then to the cuvette containing saline, increments of the platelet suspension is added until the OD matches that of the $CuSO_4$ standard. The final dilution of this portion of the platelet suspension is noted, so that the total platelet suspension can be rediluted to the same extent. One ml of this suspension will yield a platelet button containing about 0.3 mg platelet protein.

Table I

The Effect of Platelet Storage on 5HT Binding

Storage Conditions (Hours refer to time elapsed after blood withdrawal)	5HT Bound (μg 5HT/mg platelet protein)
Blood pooled, button prepared and incubation begun at 12 hrs.	2.18
Blood in individual tubes, refrigerated overnight; pooled button separated and incubated at 24 hrs.	2.13
Blood pooled, platelet button prepared at 12 hrs; dry button refrigerated. Incubation at 24 hrs.	1.44
Blood pooled, button prepared at 12 hrs; button suspended in incubation saline and refrigerated overnight. Incubation at 24 hrs.	0.74

Table II

The Effect of Platelet Storage on 5HT Binding by Platelets in the Saline Control and in the Presence of Platelet-Antibody Containing Serum

Storage Conditions (Hours refer to time elapsed after blood withdrawal)	5HT Bound by Saline Control (Antibody Free) (%)	Difference Between Saline Incubation and that Containing Serum with Platelet Antibodies (%)
Blood refrigerated in individual tubes; pooled and platelet button prepared at 24 hrs.	100 (by definition)	68
Blood pooled and platelets harvested, dry button refrigerated at 12 hrs.	64	15
Blood pooled, platelets harvested and suspended in plasma, refrigerated at 12 hrs.	58	3
Blood pooled and refrigerated at 12 hrs; platelets harvested at 24 hrs.	33	4
Blood pooled, platelets harvested, suspended in incubation saline and glucose, refrigerated at 12 hrs.	32	4

D. Storage of Platelets and Serum for 5HT Analysis and Binding Studies

1. Storage of Platelets for 5HT Binding Studies

For this investigation, the most plentiful source of platelets results from pooling tubes of patients' blood taken each morning (at "0" hour) for routine laboratory investigation. However, this blood is not available for platelet studies for another 8 hours, during which time it stands at room temperature. Thus it is necessary to determine the most satisfactory method for overnight storage of platelets.

In Table I is shown the effect of the storage on the 5HT binding ability of platelets (C'- and calcium-free medium). Data in Table II shows not only the effect of storage on the binding ability, but also the reduced ability of the platelets to exhibit a significantly lowered uptake in the presence of antibody-containing serum (C' and calcium present). Thus, it is apparent that the best method of obtaining viable platelets for 5HT uptake studies is to refrigerate the blood in individual tubes overnight, pool the blood the next morning (i.e. at 24 hours) and separate the platelets and carry out the 5HT uptake study as a continuous process.

2. Storage of Serum Suspected to Contain Platelet Antibodies

Antibody-containing serum for platelet antibody tests yields consistent 5HT binding results when kept at -20^{o}C over a minimum period of one month. Samples also withstand thawing and refreezing.

3. Storage of Platelets for 5HT Assay

For platelets awaiting assay of 5HT, no satisfactory storage method is known. Storage of whole blood, as well as the storage of platelets after their separation from whole blood and their solution in 0.02 N HCl is unsuccessful. The storage of the platelet button at -20°C is likewise not satisfactory. Cryogenic temperatures may solve this problem, but this is not pertinent to this investigation as its purpose is the development of methods suitable for routine investigation of thrombocytopenia.

4. Storage of Serum for 5HT Assay

It may be possible to measure platelet 5HT by using clotted blood so that the platelet 5HT is released into serum. Such serum may be stored at -5°C for at least two weeks without a significant change in 5HT value.

E. Platelet Solvents for 5HT-C^{14} Counting and Platelet Protein Analysis

Because acidic solutions cause quenching in liquid scintillation counting, the need arose to replace the 0.02 N HCl platelet solvent used in preparation for platelet 5HT analysis by fluorescence. Since it is necessary to express 5HT binding data in terms of mg of platelet protein, the solvent must also be suitable for the quantitation of protein.

1. 5N Sodium Hydroxide

As 5N NaOH has a limited solubility in the Dioxane Liquid Scintillator (DLS) to be used, a method of paper strip impregnation with the platelets dissolved in 5N NaOH was tried.

Platelet-NaOH solution (0.1 ml) is air-dried on a 2 x 3 cm strip of Whatman #1 filter paper, which is then placed in the counting vial and immersed in the DLS. When counted, an efficiency of about 65% is achieved. If the paper strip is removed from the vial within 5 hrs., the elution of radioactivity into the DLS is under 3% of that originally present. If there could be some pooling and re-use of DLS, a substantial financial saving could be realized. Thus 5N NaOH is a suitable and economical solvent for radio-active assay of platelets.

2. Sodium Lauryl Sulfate (2 gm/100 ml)

Solution of the platelet button in sodium lauryl sulfate (SLS) was also investigated, and found to be the method of choice.

After a platelet button is dissolved in 1.0 ml of 2% SLS, 0.1 ml is removed to a counting vial, and 15-20 ml DLS is added. The counting efficiency of C^{14} in this mixture is 74% (71%-77%). Thus SLS facilitates a simpler method of radio-active assay, the efficiency is improved, but there is no possible reutilization of DLS.

SLS also facilitates a simple protein assay. When 0.1 ml of a protein-SLS solution is added to 3 ml of water, the peak OD is below 200 mμ, which is not a suitable analytical wavelength. However, a good Beer's Law plot is obtained when the OD is read at 203 mμ. The values for platelet protein con-centration in 2% SLS when calculated from the OD readings at 203 mμ, compare well with results obtained by the Folin-Phenol method (Section III.G.1.). As little as 0.01 mg of protein can

be determined by either method.

F. Quantitation of 5HT

1. Fluorometric Analysis of Platelets

This method is that used by Weissbach and Redfield (20). Prepare a blank and standard by pipetting 1 ml of 0.02 N HCl, and 1.0 ml 5mg% 5HT in 0.02 N HCl, into the respective tubes. For each unknown, pipet into a tube 1.0 ml aliquots of the platelets dissolved in 3 ml 0.02 N HCl.

To each tube, add 1.0 ml deionized water, 0.2 ml 10% $ZnSO_4$ and 0.1 ml of 1N NaOH. Mix well, centrifuge at 3,000 r.p.m. for 10 minutes. The clear supernatant is assayed fluorimetrically; excitation 303 mμ; fluorescence 338 mμ (uncorrected). By this method normal individuals were found to contain 0.1-0.4 μg 5HT/mg platelet protein.

2. 5HT-C^{14} Quantitation by Liquid Scintillation Counting

Dissolve the platelets in 1 ml 2% sodium lauryl sulfate (SLS). Pipet 0.1 ml into a liquid scintillation counting vial. Add 15-20 ml Dioxane Liquid Scintillator (DLS) (Nuclear Enterprises #220). The solution is counted, the background subtracted and the C^{14} present calculated by comparison with a similarly treated standard containing 0.2 μg 5HT-C^{14} in 0.1 ml 2% SLS.

3. Fluorometric Determination of Platelet 5HT After Its Release into Serum

In oral 5HT loading trials, it is preferable to obtain at least one daily assay of 5HT. Since it was found unsatis-

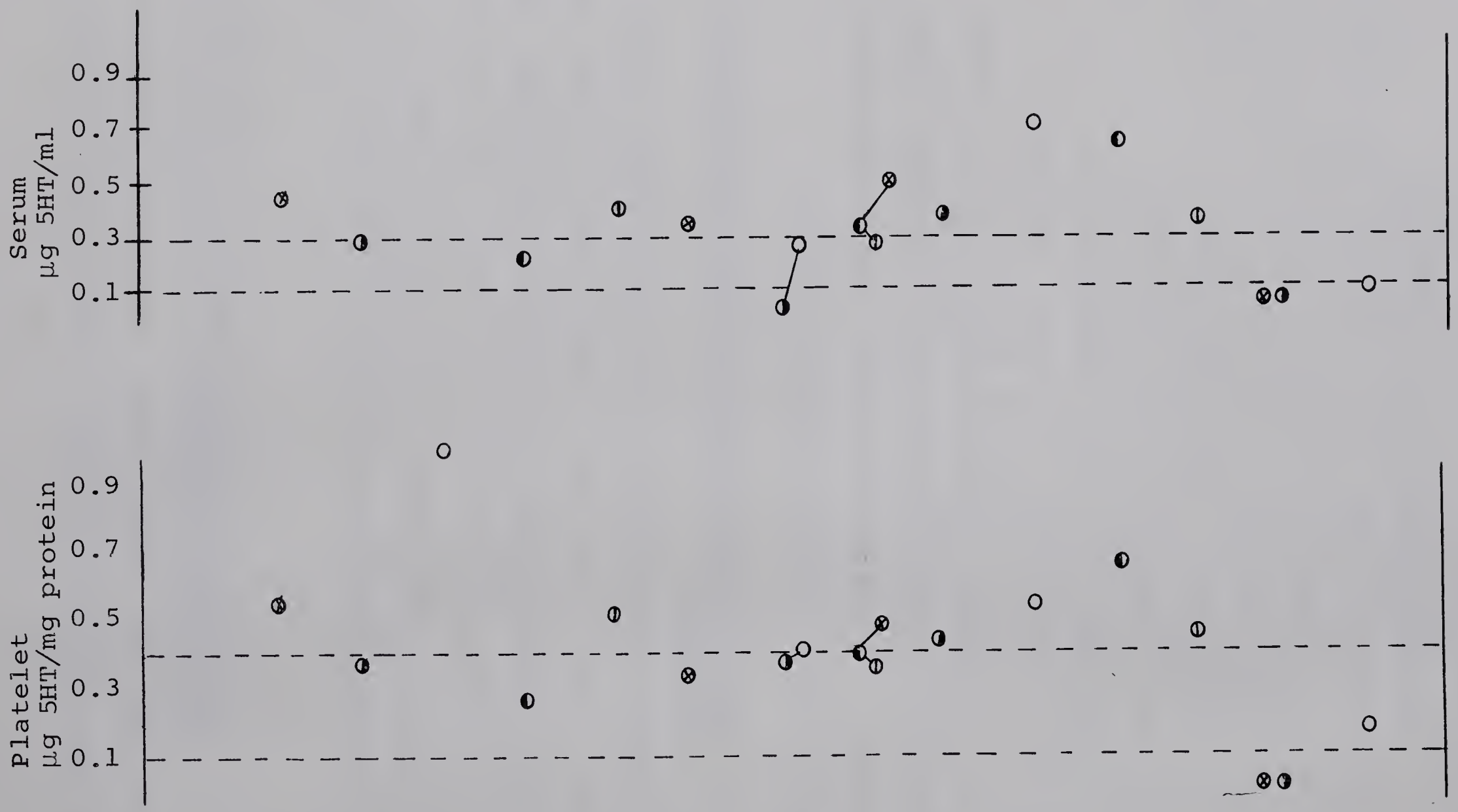

Fig. 2. A comparison of platelet and serum 5HT values.
(Note): The area between 0.1 and 0.4 µg/mg; and 0.1 and 0.3 µg/ml represent a proposed "normal" range. Connected points (o-o) represent determinations on separate specimen from one patient.

factory to store platelets awaiting 5HT assay (Section III.D. 3.), an alternative, that of assay of 5HT after its release into serum, was studied.

A modification of the method described by Ashcroft (21) gave promising results. To 0.5 ml serum is added 2 ml deionized water. Then 0.5 ml 10% $ZnSO_4$ is added and the tube contents are immediately mixed well. Rapidly add 0.05 ml 5N NaOH and shake vigorously. The protein precipitate is allowed at least 5 minutes to form, then the tubes are centrifuged. Remove 2.0 ml of the clear supernatant to another tube. Immediately before reading, 0.5 ml concentrated HCl is added to the supernatant. The resulting fluorescent emission is read; activation 300 mμ, fluorescence 550 mμ (uncorrected). By this method, sera of normal individuals were found to contain 0.1-0.3 μg 5HT/ml.

A comparison of the two fluorimetric methods is shown in Fig. 2. The area between the dotted lines on both the upper and lower graphs represent the proposed normal range for each method. It can be seen that in most cases there is a good correlation between the 5HT levels in the platelets (which were assayed immediately after withdrawal of the blood) and 5HT liberated into serum from the platelets after clotting. The latter values were obtained on sera stored at -20°C as long as two weeks.

G. Quantitation of Platelet Protein

1. The Folin-Phenol Method

This method for protein determination is a micro method adapted from Sutherland's(25), for use in the analysis of

platelet protein.

The platelet button (1-2 mg platelet protein) is dissolved in 3 ml 0.02 N HCl. To a 0.10 ml aliquot of this solution, add 0.4 ml 0.02 N HCl. Prepare the blank by substituting 0.5 ml 0.02 N HCl; and the standards by using 0.5 ml aliquots of a commercial control sera (Versatol), which has been diluted to contain 0.04, 0.08, 0.12 mg protein, respectively.

To each tube add 5 ml alkaline copper solution (1 ml 2% $CuSO_4.5H_20$, 1 ml 4% sodium tartarate, diluted to 100 ml with 4% Na_2CO_3). Incubate for 10-15 minutes at 45°C. Add to each tube 0.5 ml Folin-Ciocalteau Phenol reagent (Fisher Scientific Co.), freshly diluted 1/4 with water, and mix immediately and thoroughly. On standing at room temperature for 30 minutes, a blue color develops which may be read at 740 mμ, against the blank. The protein standard curve is not linear but is analytically useful as long as the standard values bracket the platelet protein values.

2. Spectrophotometric Analysis

A well-drained platelet button, obtained from 1-2 ml blood or spun down from a 5HT uptake study is dissolved in 1 ml 2% sodium lauryl sulfate (SLS), and 0.1 ml of this solution is mixed with 3 ml deionized water. Similarly, prepare a blank containing 0.1 ml SLS and a standard using a commercial control serum which is diluted so that 0.1 ml contains 0.04 mg protein.

The OD of the standard and tests is read at 203 mμ, using the blank to zero the spectrophotometer.

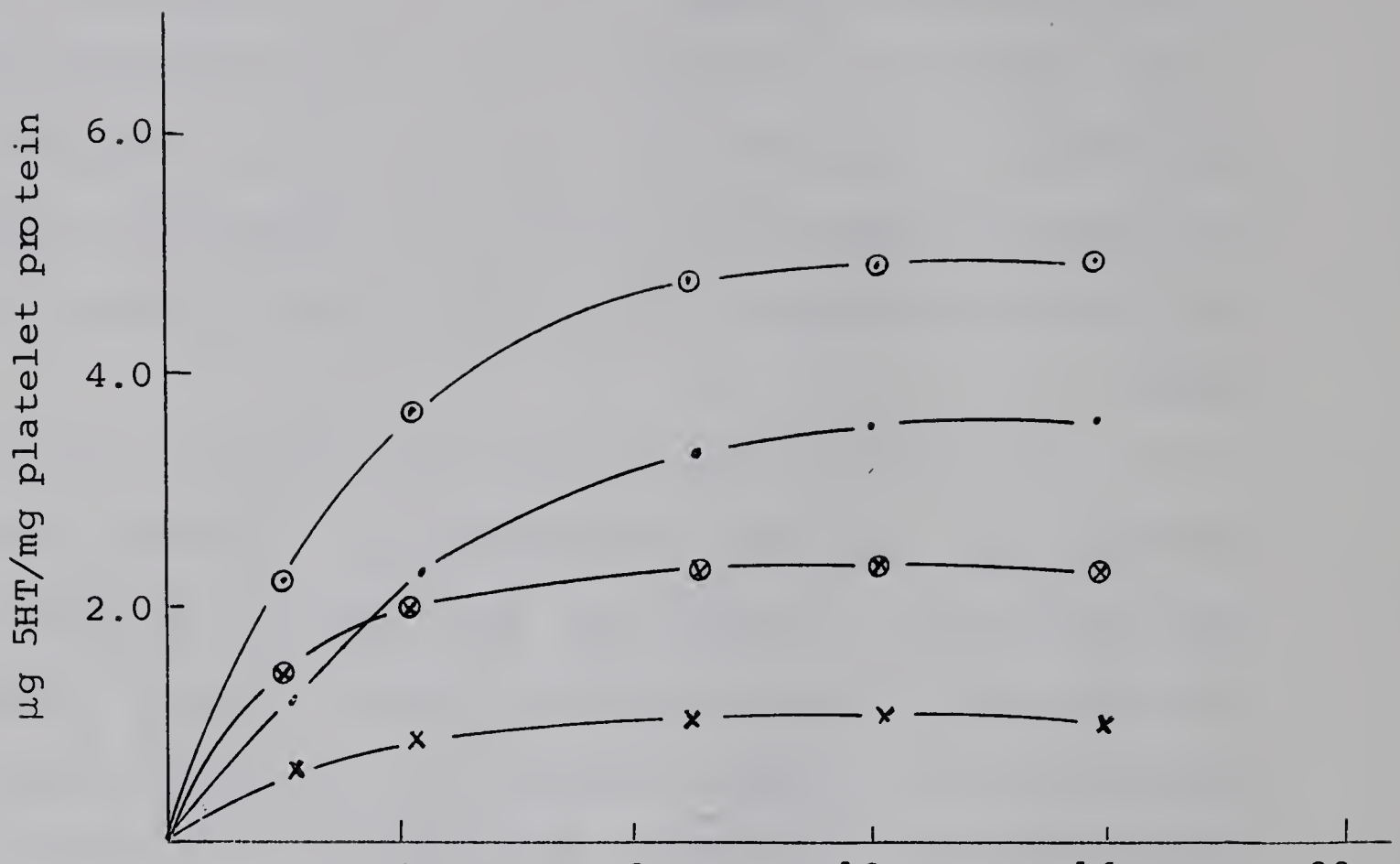

Fig. 3. Binding of 5HT by platelets in plasma vs. saline media.

(Note): Incubation of fresh platelets in saline —⊙-
Incubation of fresh platelets in plasma —⊗-
Incubation of pooled platelets in saline -•-
Incubation of pooled platelets in plasma -×-

H. Methods for Studying Uptake of 5HT by Platelets

1. Introduction

The original incubation mixture studied was similar to that used by Bridges (7), resulting in a 1/12 dilution of a test serum in the final incubation.

The proportions used are: 0.2 ml test serum, 1.0 ml of platelets (suspended in plasma or in the salt solution described in Section IV.C.1.); 0.2 ml, 0.308M glucose and 0.2 ml, 5.0 mg% 5HT-C^{14}. Ingredients are mixed by inversion and tubes incubated at 37°C.

Figure 3 shows results obtained for both "new" platelets (from a single donor) and "pooled" platelets. The latter were harvested from blood collected 8-24 hrs. earlier, the former from blood freshly collected by the method for collection for platelets for 5HT assay (see Section III.B.). The maximum uptake on incubation is not observed until after 8 hours. By this time, fresh platelets had bound 4.5 μg 5HT/mg platelet protein. Since platelet button contains slightly over 1 mg of protein, about 50%, therefore, of the available 5HT had been bound. The pool platelets had bound 2.5 μg 5HT/mg protein.

The concentration of platelets was varied 4-fold, to determine whether the uptake of 5HT by pooled platelets in saline incubation would be affected. There was no significant variation in binding in terms of μg 5HT/mg platelet protein.

Regarding the suspension medium for the platelets, it is obvious from Fig. 3 that both pooled (old) and fresh platelets exhibit a greater uptake of 5HT in a saline than in the plasma medium. Although the uptake with fresh platelets is

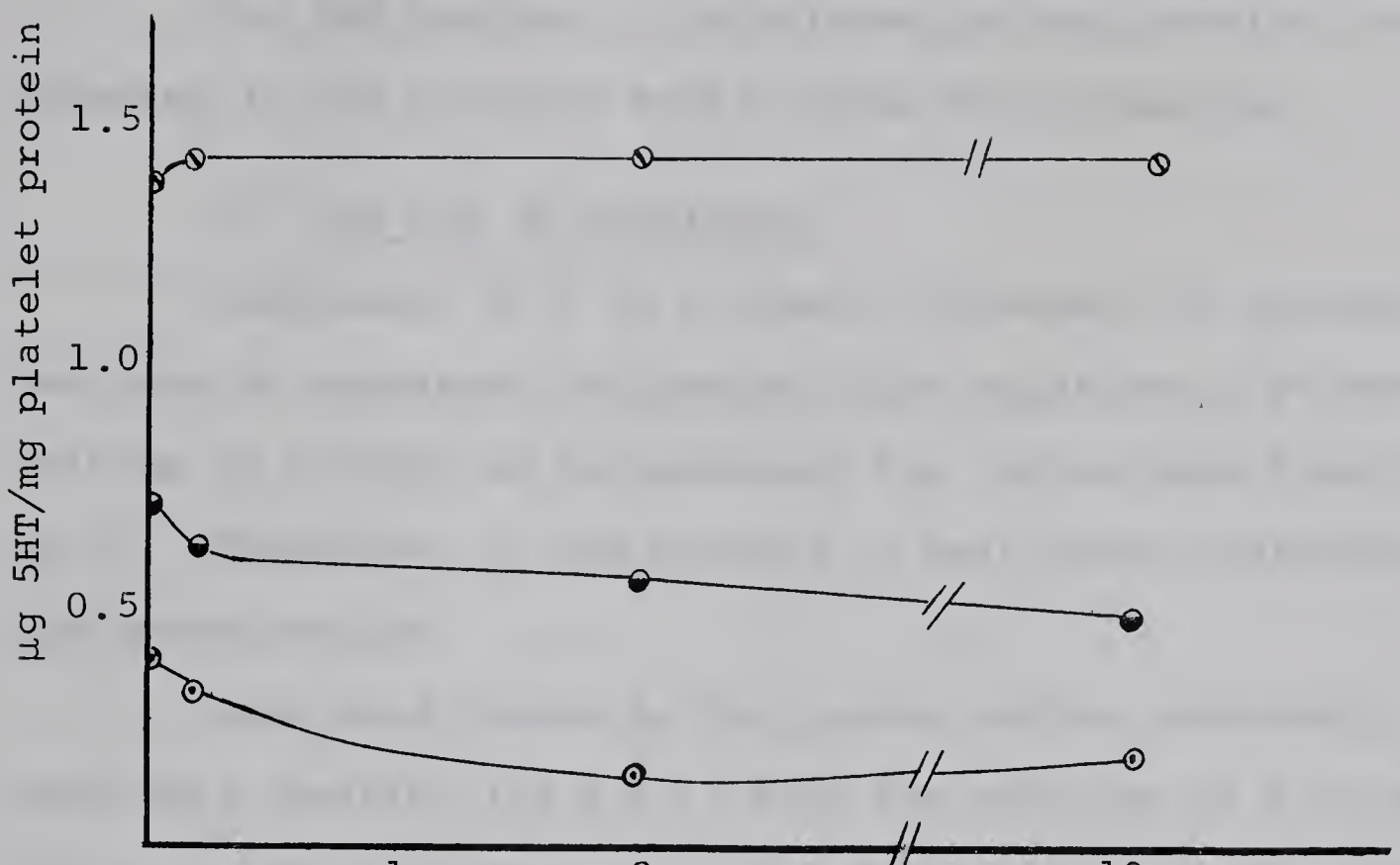

Fig. 4. The effect of complement (and calcium) on 5HT binding by platelets.

(Note): Incubation in the presence of normal human serum —⊘—.
Incubation in the presence of human sera clinically suspected to contain antibodies —◒—.
Incubation in the presence of anti-human platelet serum from rabbit —⊙—.

greater, pooled platelets are more readily available. Therefore, pooled platelets, in saline medium, are used in future work.

The 5HT binding is calculated per mg platelet protein detected in the platelet button after the incubation.

2. The Use of Complement

Complement (C') is a common ingredient of systems designed to encourage antigen-antibody reactions. Further, calcium is thought to be necessary for the optimum function of C'. Therefore, it was decided to test these additions to the uptake medium.

Sera were tested by the uptake method previously described (Section III.H.1.), with the addition of 0.25 mg $CaCl_2$ (0.1 ml, 250 mg% $CaCl_2$), and varying amounts of complement (C'). The C' was diluted such that 2 units are present in 0.1 ml. Three sera were tested: (1) normal human serum (antibody-free); (2) patient sera containing clinically suspected antibodies, and (3) anti-human-platelet serum from a rabbit injected with platelets in adjuvant.

The results of the uptake study are shown in Fig. 4. In the C' free system, there is a inhibition of 5HT binding in the presence of suspected antibodies. However, the dimension of the difference in uptake between the antibody-containing sera and the normal sera is significantly enhanced by the addition of C'.

In order to rule out the possibility that the value of C' in the test was due to a non-specific action, a test was

run in which the effect of heat-inactivated C' was compared to a C'-free system and to a system containing active C'. Indeed the results when inactivated C' (i.e., heated to 56°C for 20 minutes) was added to either serum-free or test systems did not significantly differ from those of the C'-free systems.

Thus it was decided to add 2 units of active C' and 0.25 mg $CaCl_2$ to the incubation medium.

3. Addition of Adenosine Triphosphate (ATP)

Since, as previously mentioned, 5HT binding is an active process, it was possible that the supply of ATP was a governing factor. However, the addition of ATP to saline control and test media did not enhance the uptake of 5HT by pooled platelets nor assist in the differentiation of anti-body-containing serum. There was no further investigation concerning the use of ATP in this system.

I. Anti-Human Platelet Sera from Rabbits

1. Summary of Rabbit Injection Program

In order to have a plentiful supply of platelet anti-body-containing serum, rabbits are immunized by injection of human platelets.

Injections contain platelets harvested from 20 ml of blood. The button is suspended in approximately 1 ml of sterile normal saline or 1/2 ml of adjuvant (Complete Freund Adjuvant, Baltimore Biological Laboratory), plus 1/2 ml sterile saline. For the latter, mixing must be vigorous and can be

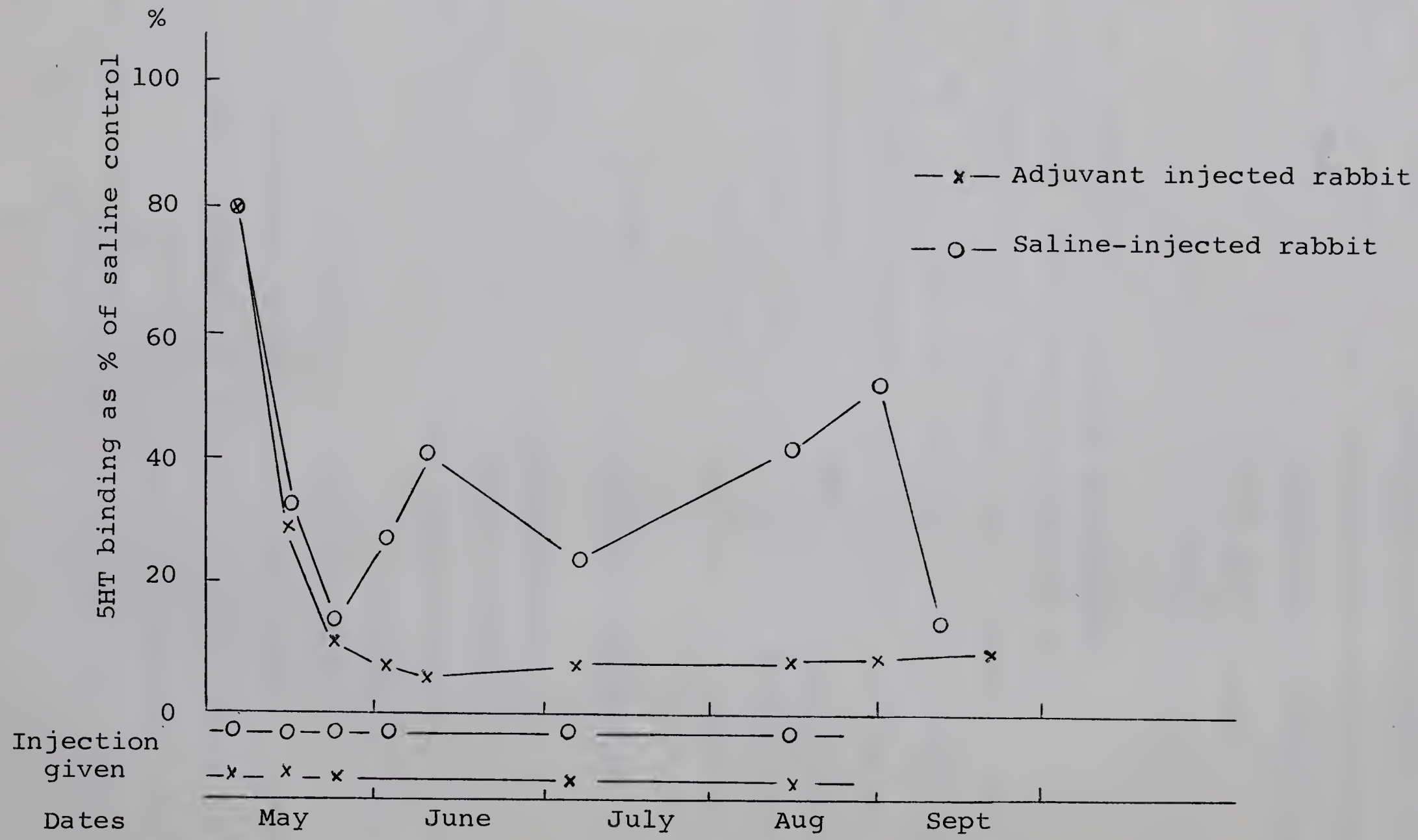

Fig. 5. The development of anti platelet-5HT-binding factor in rabbit sera, as a function of injection pattern.

accomplished by drawing the mixture repeatedly through an 18 gauge needle with a syringe (24). The 1 ml suspension for each rabbit is injected subcutaneously at the shoulders and hips (4 sites). Blood is then taken from the rabbit by cardiac puncture. Up to 50 ml may be taken in 1 week.

Four rabbits were used. Two were injected with platelets suspended in adjuvant. The others were injected with platelets suspended in saline. A series of four weekly injections were planned for each rabbit; however, the adjuvant injections were discontinued after the third week, as the rabbits had developed excessively large lumps at the injection sites. The saline-injected rabbits had the series of four injections, not having developed the problem initially. The lumps on the saline-injected rabbits remained through the four months of the study, however, while those on the adjuvant-injected rabbits decreased markedly. All the rabbits remained in apparently good health.

To assess the development of antibody, the rabbit sera was checked for its ability to inhibit the uptake of 5HT by normal human platelets. Because of the high antibody titer, a 1/15 dilution of serum was used (Section IV.C.), and the results of this study are plotted in Fig. 5.

The rabbit injected with platelets suspended in adjuvant appeared to have developed the antibody to a greater degree than the saline-injected rabbit. Thus, using an immunological technique, the anti-human-platelet-5HT binding factor, antibody, was successfully developed in rabbits.

It is further demonstrated that normal rabbit sera did not effect the uptake of 5HT by human platelets (Fig. 6). If the uptake in absence of any sera (saline control) is defined as 100%, the uptake when normal rabbit sera is present, at 7 hours is 95%. However, a markedly diminished uptake (20%) is shown by platelets incubated in the presence of serum obtained from a rabbit after the platelet injection program.

J. Titration of Antibody-Containing Serum

The "titer" is an expression of the strength or concentration of an antibody in serum. Using platelet-5HT-binding inhibition, a similar determination can be made by a series of incubations testing increasing dilutions of the serum. Traditionally, titer is reported as the highest dilution in which the effect of the antibody can be noted.

An effective serum dilution of 1/3, 1/15, 1/30, 1/60, 1/240 ... can be obtained for titration by using the 1/3 serum dilution method (Section IV.C.1), the 1/15 serum dilution method (Section IV.C.1), and the 1/15 serum dilution method replacing the 0.1 ml serum with 1/2, 1/4, 1/16 ... dilution of serum in normal saline.

In Fig. 7 are the results of such a titration on the anti-platelet-5HT-binding factor in injected rabbit serum. The titer is 1/60.

It is possible that 1/90 is a slightly better indication of titer. This figure is derived by extending the straight line made by joining the first four points, until it intersects

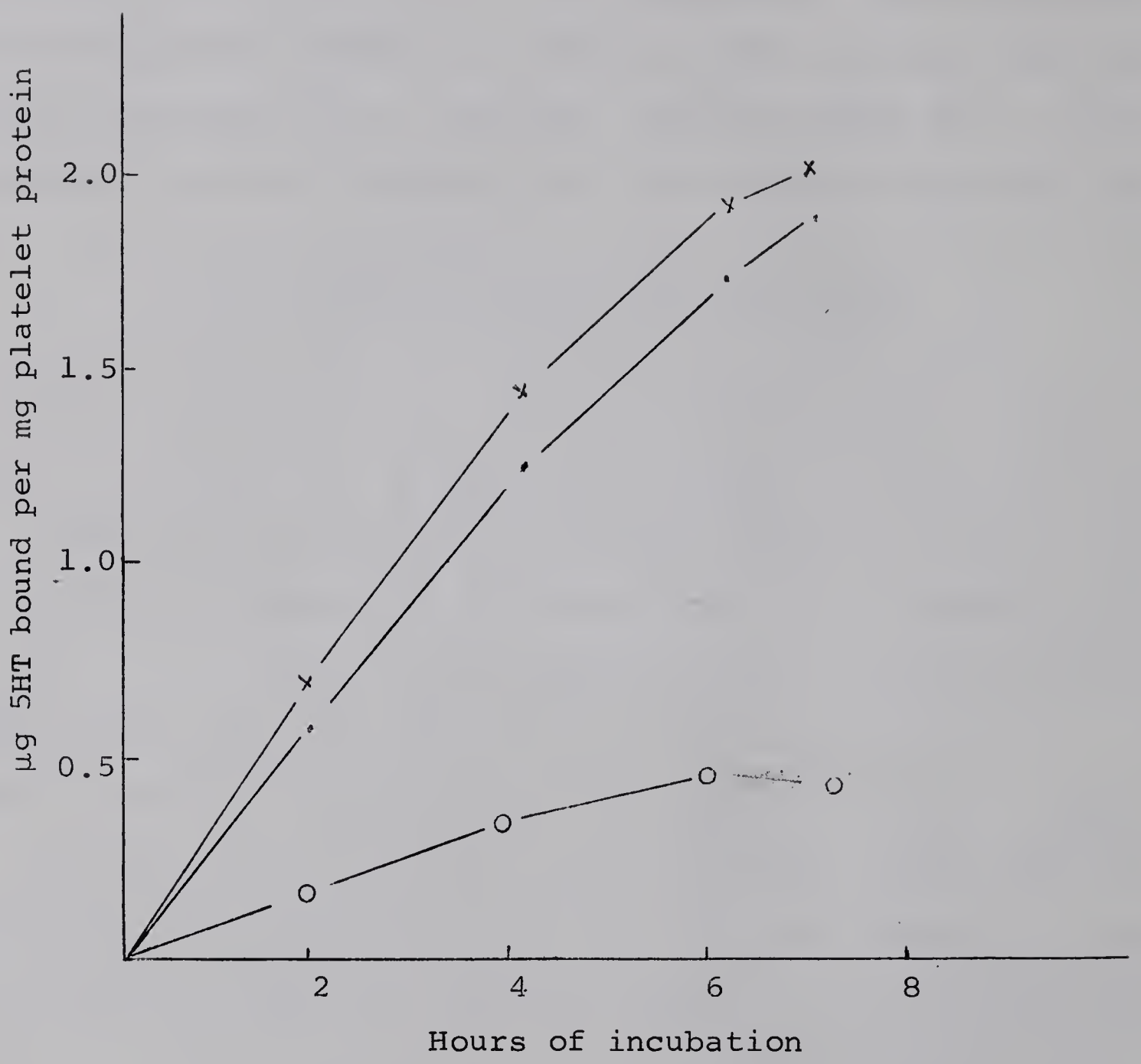

Fig. 6. Platelet uptake of 5HT in the presence of normal and injected-rabbit sera.

(Note): Complement free system, 1/12 dilution. At 7.5 hrs. incubation; if saline control -x- binding = 100%, normal rabbit sera -·- permitted 95% binding, and rabbit antibody-containing serum -o- allowed 20% binding.

an imaginary line, perpendicular to the "y axis" at 80% (the lower limit of "normal" binding; see Section IV.C.2.). The value on the "x axis" at this point is 1/90.

To emphasize that the "titration" is not simply a dilution of some incidental component of rabbit serum, control studies were done using the serum taken from a normal (non-injected) rabbit. At each serum dilution, the platelet 5HT binding was within the normal range (80-100% of the saline control). This indicates once again that the anti-platelet-5HT-binding factor is present in rabbit serum as a result of an immunological process and is therefore an antibody.

K. Absorption Studies

Absorption, the removal of antibodies from serum by allowing them to combine with the respective antigens, is a method commonly applied in the immunological study of red blood cells. It was therefore decided to try to apply this immunological tool to the anti-platelet-5HT-binding factor. Several methods were tried, the most effective one being the following:

Platelets harvested from 40 ml human blood are equally divided by aliquoting the final wash suspension (see Section III.A.B.C.) into four incubation tubes, then continuing with the final centrifugation. After the wash liquid is decanted in the normal manner, the platelets in tube 1 are suspended in 1 ml of the serum to be absorbed. The tube is incubated 1 hour at 37^{o}C, and the platelets removed from the serum by centrifugation. The serum is decanted into tube 2 and these platelets suspended and incubated as in the first absorption.

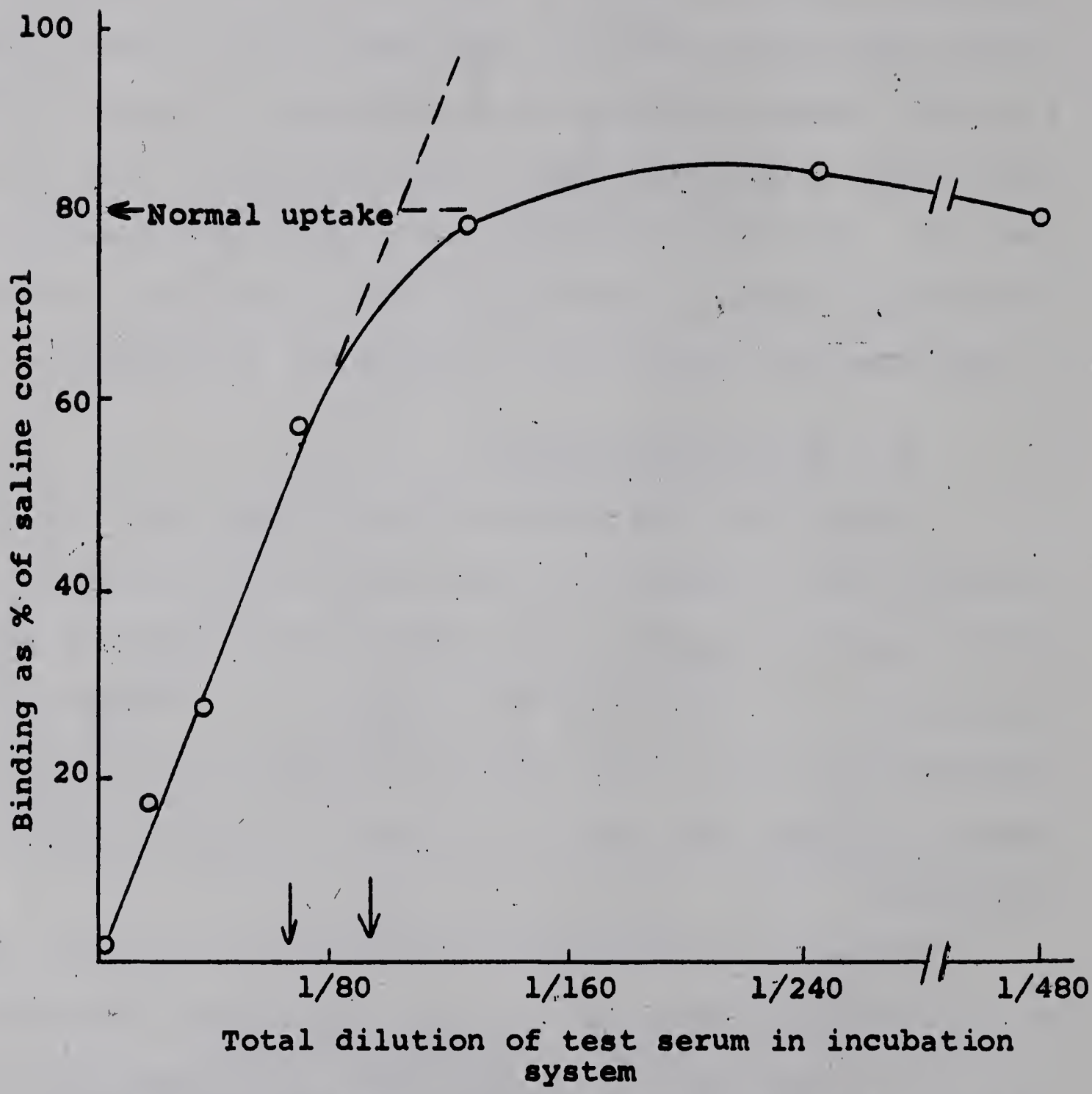

Fig. 7. Uptake of 5HT by human platelets in the presence of serial dilutions of anti-human-platelet rabbit serum.

The absorption is repeated four times in all.

The absorption was assessed by a 5HT uptake test, using a 1/15 dilution of serum (Section IV.C.1.), and the results are expressed using the 4+ grading system described in Section IV.C.2. Absorption tests were conducted on normal rabbit serum (antibody-free), a patient serum (clinically suspected to contain platelet antibodies), and serum from a rabbit after a series of platelet-in-saline injections were tested.

As may be expected, the absorption had little effect on the normal rabbit serum. Results of the assessment of the absorption on the two antibody containing sera are found in Fig. 8.

The serum of patient AB. exhibited only slight removal of 5HT uptake inhibiting factor, although in terms of the grading system used, there was a decrease in the strength of the antibody from 3+ to 2+.

Two independent absorptions of serum from the saline injected rabbit were made. The initial absorption was continued 9.5 hours, but with no exchange of platelets. The result is that 5HT binding increased from 28% to 55%, or in terms of antibody strength, decreased from 3+ to 2+.

However, when four 1-hour absorptions were used, the 5HT binding in the subsequent test increased from 35% to 88% of the binding observed in the saline control. Thus, the serum which was originally graded 3+ for antibody strength became "negative" when absorbed in this manner.

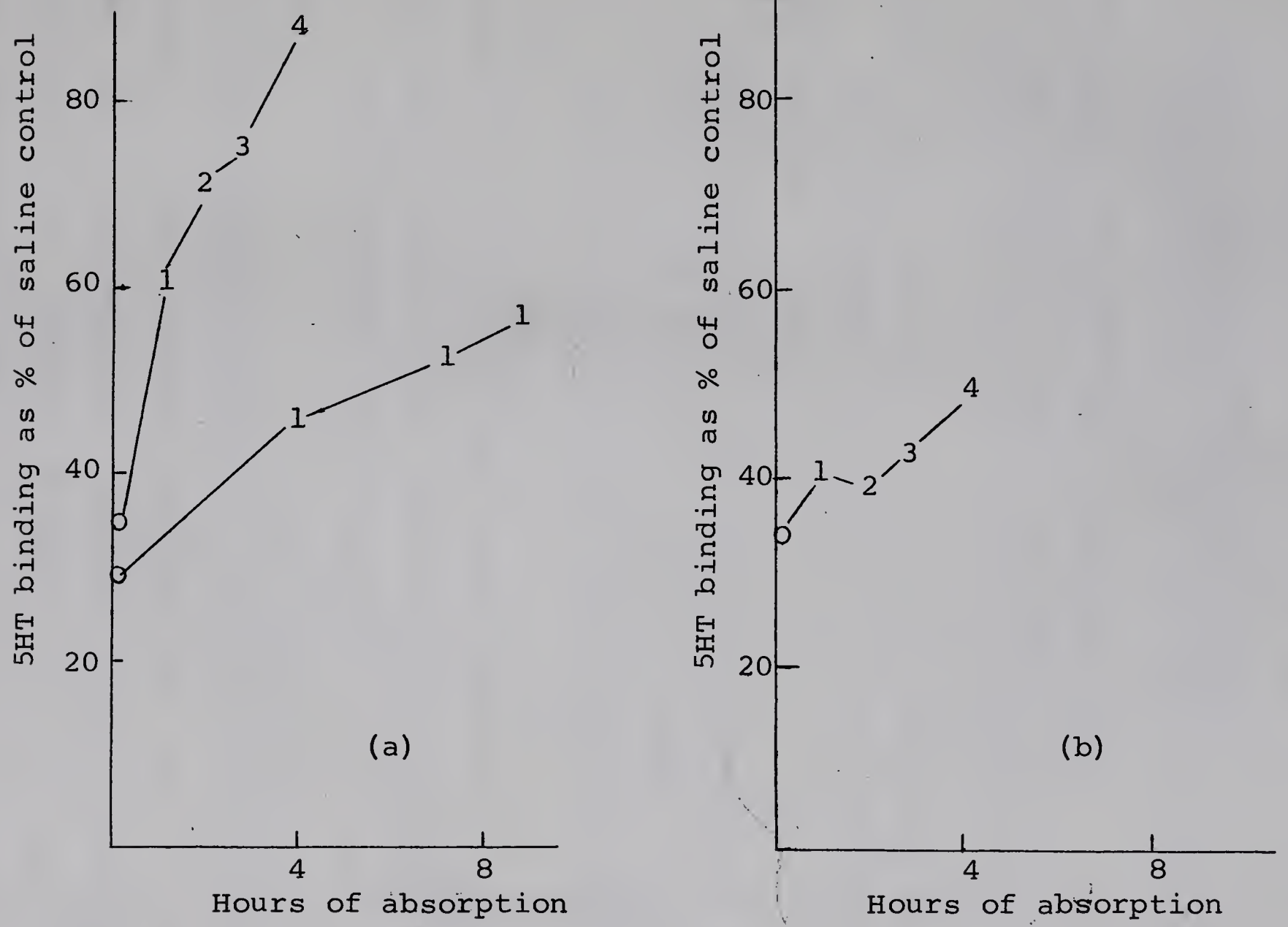

Fig. 8. The effect of absorption on anti-platelet-5HT-binding factor.
(a) rabbit anti-human-platelet serum;
(b) human serum (patient BA)
(Note):Figures -x- indicate number of absorptions.

To conclude, multiple absorptions with human platelets essentially removed all of the anti-platelet-5HT-binding factor from immunized rabbit sera. However, a single experiment on human sera, containing anti-platelet-5HT-binding factor yielded inconclusive results.

IV. SUMMARY OF METHODS USED FOR PLATELET STUDY

A. Platelet 5HT

This method was used for measuring platelet 5HT after oral administration of 5HT and also for stability studies of 5HT in stored platelets.

Blood (about 7 ml) is collected, and the platelet button is prepared at 5°C as outlined in Sections III.A.B.C. After solution of the button in 3 ml 0.02 N HCl, the platelet 5HT is assayed fluorimetrically (see Section III.F.1.), and the protein is assayed by Sutherland's modification of the Folin-Phenol Method (Section III.G.1.).

The result is expressed: µg 5HT/mg platelet protein. Normal levels in adults were found to be between 0.10 and 0.40 µg 5HT/mg. The standard deviation, determined using a series of 23 patients (46 tubes of blood) was ±0.035 µg 5HT/mg platelet protein. (Blood was aliquoted immediately on being withdrawn from the patient, into 2 tubes which were treated in parallel fashion through the platelet separation, 5HT and protein assay.)

B. Method for Quantitating Urinary 5 Hydroxyindole Acetic Acid (5HIAA)

The metabolism of 5HT by deamination and oxidation results in production of 5HIAA, and this end-product is excreted in the urine. The following assay is a method to attempt

to confirm that the orally administered 5HT, or at least its metabolite, is indeed finding its way to the blood.

The method, an adaptation of one used by Chadwick (23) is as follows. Make a 1/20 dilution of test urine in water. Prepare an internal standard by dissolving approximately 1000 µg 5HIAA in 50 ml of diluted urine. (Cyclo-hexylammonium salt• CH_3OH of 5HIAA is used for increased solubility.) Calculate exact amount of 5HIAA present in 5 ml of the diluted urine.

For aqueous standard, similarly add 5HIAA to 50 ml water.

To 5 ml of each: 1/20 diluted urine, internal standard and aqueous standard, add 10 ml $CHCl_3$. Shake 5 minutes, spin and aspirate $CHCl_3$. To aqueous layer remaining, add 1.0 ml 6N H_2SO_4, 2 gm NaCl and 25.0 ml ether. Shake vigorously for 20 minutes and centrifuge. Remove 20.0 ml ether and add 5.0 ml 0.5 M, pH 7, buffer (dilute 50 ml of 0.5 M KH_2PO_4 and 29.63 ml of 0.5 M NaOH to 200 ml with deionized water).

Shake 5 minutes. Remove 4.0 ml of aqueous layer and add 1.0 ml 0.25% 1-nitroso-2-naphthol reagent (dissolve 0.25 gm 1-nitroso-2-naphthol in 100 ml ethanol). Prepare a blank by adding nitroso naphthol reagent to 4.0 ml buffer. Shake each tube briefly and add 1.0 ml fresh nitrous acid. Mix. Incubate at 37°C for 5 minutes for color development. Extract excess color of reagents with 2 x 5.0 ml ethyl acetate, removing the ethyl acetate layer each time after separation of the layers.

Read OD at 540 mµ against the buffer blank. The normal range is 2-8 mg 5HIAA/24 hr. urine collection.

C. 5HT Binding Test for Platelet Antibodies

1. 1/3 and 1/15 Serum Dilution Tests and Saline Control

Platelets are collected and prepared according to directions in Section III.A.B.C.

a. Reagents

(1) Incubation saline: This saline is similar to that described by Sano et al. (17). It contains per 100 ml: 10 ml 3.8% sodium citrate, 1.1 ml 0.154M KCl, 8.0 ml 0.154M Na_2HPO_4, 1.9 ml 0.154M KH_2PO_4, 0.8 ml 0.154M $MgSO_4$, and 0.154M NaCl.

(2) 5-Hydroxytryptamine-2-C^{14} (hydrogen oxalate) (New England Nuclear Corp.) 5 mg% in normal saline (.016 μc/μg).

(3) 0.308 M glucose.

(4) 25 mg% $CaCl_2$.

(5) Guinea pig complement (C') (Connaught Medical Research Laboratories). Using label information, 1 vial is freshly diluted each day so that 0.1 ml contains approximately 2 units C'. That is, after reconstitution, C' is further diluted 1/3.

b. Quantities of Sample and Reagents Used. Details of Method

For 1/15 dilution of serum in total incubation, use0.1 ml 5HT - C^{14}; 0.3 ml of a mixture of equal parts glucose, $CaCl_2$ and C' solutions (above) ; 1.0 ml of platelet suspension (Section III.C.1.); 0.1 ml of test serum.

For 1/3 dilution of test serum in total incubation, use

0.1 ml 5HT-C^{14}; 0.4 ml of equal parts of solutions of glucose, $CaCl_2$ and C'; 0.5 ml test serum, 0.5 ml platelet suspension.

Note: The method for some of the early uptake studies was C' and Ca free. The test sera was diluted to 1/12. (See Section III.I.A.).

For the saline control, to establish 100% uptake possible for each platelet harvest, simply omit the serum from the appropriate method above. No correction for volume is necessary.

Samples and reagents as described above are gently mixed by inversion if necessary and incubated 3 hours at 37° C. At the end of incubation, each tube is immediately spun at 3000 r.p.m. at room temperature. The clear supernatant is decanted and the cells washed with normal saline (see Section III.A.). After spinning again, the saline is decanted and the tubes left, inverted and refrigerated. When the incubation of all tubes is complete, 1.0 ml of 2% sodium lauryl sulfate (SLS) is used to dissolve each button.

An estimation of the 5HT bound by each button is made by C^{14} liquid scintillation counting (Section III.F.2.), and protein is assayed by the spectrophotometric method (Section III.G.2.).

2. Calculation of Results

The 5HT binding by the platelets is expressed in terms of mg platelet protein, determined for each test after incubation in order to minimize the effect of random loss of platelets during the incubation and washing procedures. The standard

deviation when 26 pairs of serum tests or saline controls were incubated and assayed, is ±0.125 μg 5HT/mg platelet protein.

The assessment of relative antibody activity, however, is based on the definition of 100% as that amount of 5HT bound per mg platelet protein in the saline control. If the 1/15 serum dilution test is used, a 4+ grading system may be applied, wherein over 80% binding in the presence of a test serum indicates that the serum is antibody-free (negative). Otherwise, the serum is graded 1+, 2+, 3+, or 4+ if the binding which occurs is 60-80%, 40-60%, 20-40%, or 0-20%, respectively. This grading system is not applicable to the 1/3 serum dilution test, as much less 5HT is bound. Indeed, in the presence of normal serum, binding is at best about 40% of that in the respective saline control.

V. A. STUDY OF IN VIVO PLATELET SURVIVAL

1. Introduction

It was thought that orally administered 5HT might be a useful tag for *in vivo* platelet survival studies. To test this hypothesis, it was planned to give 5HT in the form of serotonin creatinine sulfate, four times daily, to each of 7 individuals. Periodic assay would detect the effect of the dose on platelet 5HT concentration. After a maximum level of platelet 5HT was reached, dosage would be discontinued and frequent determinations of platelet 5HT made. The results were to be plotted on semi-log paper, and from this, the theoretical time necessary for the platelet pool to lose 1/2 of the 5HT accumulated during the loading phase (T1/2), could be calculated. Assuming that 5HT is irreversibly bound to the platelet and does not take part in platelet metabolism, T1/2 would be a reflection of the survival of the platelets in the peripheral circulation.

The determination of 5HIAA, the metabolic end-product of 5HT, was intended to indicate the effectiveness of absorption of 5HT from the gut.

The seven trials included 5 "normals" and 2 thrombocytopenic patients. In the serum of Patient AP, a thrombocytopenic, platelet antibodies had been identified by a consultant laboratory. Patient DB, a child, had acute thrombocytopenia

Table III

Results of Platelet Survival Study

Patient Identification:	EN	SK	EG	BS	MY	AP	DB
Diagnosis:	Normal					Thrombo-cytopenic	
	µg 5HT/mg platelet protein						
Initial 5HT value	.35	.31	.58	.34	.40	<.10	.11
Decline curve: Day 0	.83	.45	.87	.39	.61	.11	.30
					.58		
1	.67		.80		.55		
2			.75		.60		
3	.60				.55		
4							
5			.69				
6	.56		.68				
7			.75				
8	.42						
9			.69		.29		
10 = 20	.41				.44		
Dose received/day during loading phase (mg 5HT)	180	180	160	180	160	160	53
No. days in loading phase	17	16	19	16	27	14	7
% of dose recovered as urinary 5HIAA/24 hr. collection			87%		94%		
					104%		
T1/2 of 5HT platelet tag	4.5		5		3		

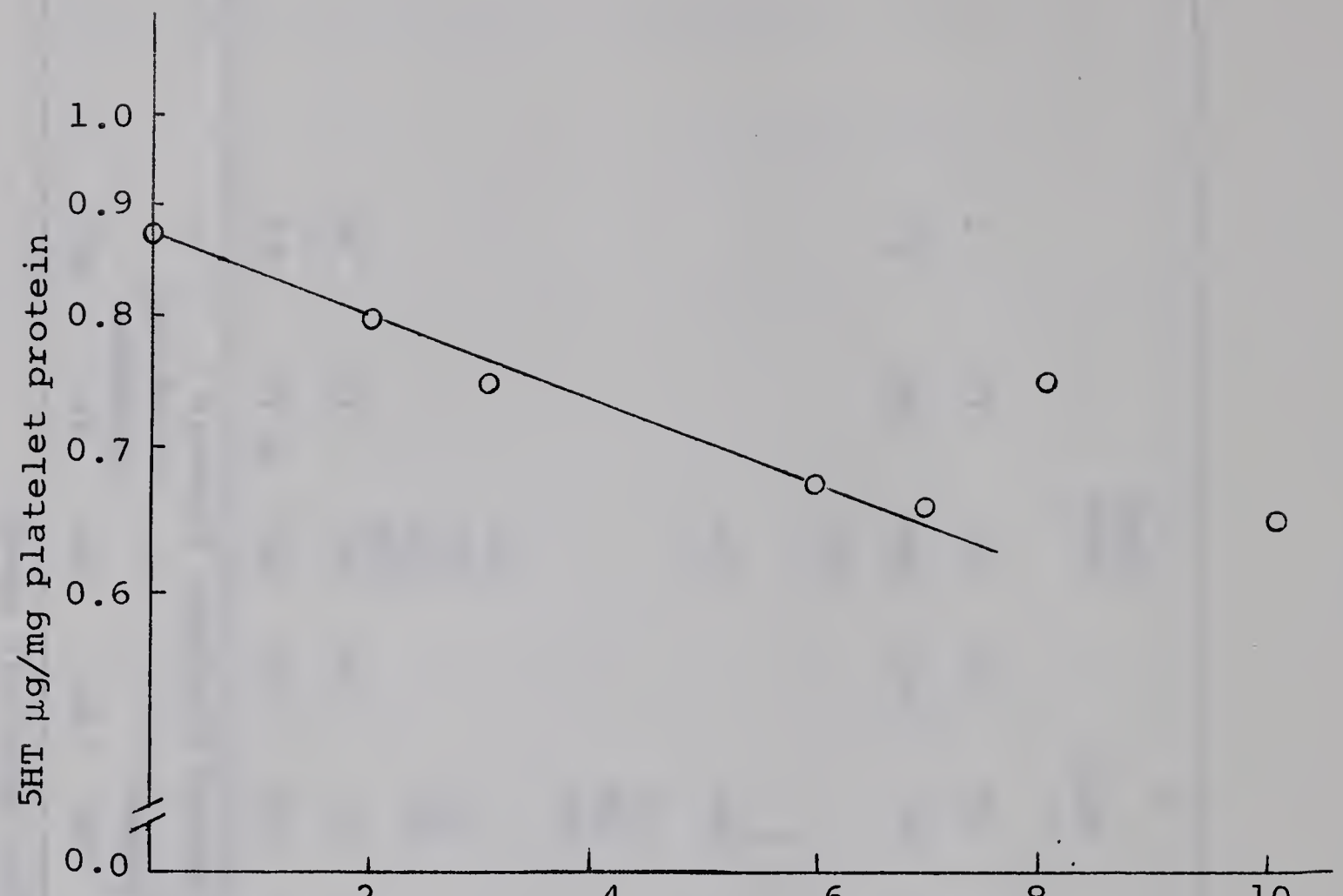

Fig. 9. Estimation of platelet survival by T1/2 of 5HT tag.

Calculation of T1/2:

$$\frac{\text{High value-Baseline}}{2} = \frac{.87-.58}{2} = .15 \ \frac{\mu g \text{ 5HT}}{\text{mg platelet protein}}$$

The time required for disappearance of .15 µg 5HT/mg platelet protein is (see graph) 5 days.

of unknown origin, followed by a spontaneous remission coincident with but not related to the administration of oral 5HT.

2. Results

In Table III, the 7 trials are summarized. Tests of SK and BS, normals, were discontinued when after 16 days, the elevation of platelet 5HT was insufficient to begin the intended determination of the 5HT decline. For the remaining three normals, the T1/2 was calculated (see Fig. 9), and found to be 3, 4.5 and 5 days. No significant increase in the exceptionally low platelet 5HT level in patient AP was achieved and the test was therefore discontinued.

The second thrombocytopenic patient, DB, was able to bind 5HT, and a significant increase in platelet 5HT was achieved. However, due to the spontaneous remission of the thrombocytopenia, the patient was discharged before the T1/2 value could be determined.

3. Conclusion

A T1/2 for platelet survival of 3.5 to 5 days calculated from the studies on three normals is compatible with published estimates of platelet life span and the results of survival studies using other techniques (2,3). However, there are several disadvantages to this method. In particular, it is evident that normal individuals vary widely in the platelet 5HT levels they are able to achieve. Recently, Melmon (22) has confirmed that this difference exists, "due to pharmacogenetic variation between normals". Secondly, a procedure requiring two weeks of dosage of 5HT is too lengthy in terms

of hospitalization time. Further, the platelets of a thrombocytopenic (patient AP), whose serum probably contained platelet antibodies, failed completely to respond to the oral administration of 5HT. Since the primary purpose of platelet survival studies would involve investigation of thrombocytopenia in the presence of platelet antibodies, there was little value in proceeding with the proposed method. However, the possibility that antibodies were responsible for the failure of oral 5HT to tag the platelets in vivo, led to the major phase of this research, that involving an in vitro detection of platelet antibodies by detection and measurement of inhibition of platelet 5HT binding.

B. PLATELET 5HT BINDING TESTS AS A MEANS OF DETECTING PLATELET ANTIBODIES

1. Introduction and Tables

The sera of over 50 individuals were tested for the presence of platelet antibodies by at least one of two related methods, the 1/15 serum dilution test which contains complement (C'), and the 1/12 serum dilution test (C' free). Another method, 1/3 serum dilution (with C') was also tried. The 1/15 serum dilution method has been found the most useful and will therefore be used to evaluate the other two.

As it was originally anticipated that this platelet 5HT binding test be a means to document the presence of isologous platelet antibodies in serum, the evaluation of the method was based on two major categories of sera; that from transfused individuals, and that of non-transfused individuals.

Care was taken to include in the former category sera of individuals who for any reason had received over 10 units of blood. (It is presently accepted that the patient who receives over 10 units of blood in an unspecified period will develop isologous platelet antibodies.) Care was also taken to include thrombocytopenic patients in both categories.

The results for the 18 transfused patients are found in Table IV. The non-transfused group of 34 individuals which was tested to ascertain the specificity of the procedure, included 12 normal volunteers and 8 patients whose disease was not of a hematological nature. These 20 individuals had normal uptake of platelet 5HT. The remaining 14 individuals were thrombocytopenic and/or had positive results reflecting apparent antibody activity. Results on this group are shown in Table V and a discussion of their significance is in Section V.B.3.

2. The Detection of Antibodies Following Blood Transfusions

It is well-known that after many transfusions, patients develop isologous platelet antibodies. This, however, may be clinically insignificant until the patient receives a further transfusion, at which time the isologous platelets are destroyed. By available methods, the presence of the isologous platelet antibodies may be undetectable. A study was made on patients having received transfusions to see if the anti-platelet-5HT-binding factor (antibody) could be demonstrated in their sera. The results shown in Table IV

Table IV

Results Obtained on Sera of Transfused Individuals

Patient	Sex/Age	Medical History	Results 1/12 Serum dilution test (C' free)	1/3 Serum dilution test (C')	1/15 Serum dilution test (C')
AK	F/68	In past 18 mos., 26 units blood. Platelets decreased. Coombs test for platelet antibodies negative. Multiple myeloma.	+,neg.	7%	4+,3+
BB	F/19	In past 18 mos., 30 units blood and packed cells. Platelets about 15,000/mm^3. Aplastic anemia.	2+,3+,(3+)	-	-
BA	F/34	Transfused frequently since 1957. Febrile reaction to whole blood transfusion 1963, given packed cells 1965,1966. Leukoagglutinins, detected. Platelets 149,000/mm^3. Discharge diagnosis G.I. bleeding.	-	1%	3+
AR	M/59	Received 8 units blood and packed cells in past 2 mos. Multiple myeloma. Platelets 60,000-139,000/mm^3.	1+	-	-
HG	M/37	Received 6 units blood on 2 days within past 10 days. Chronic myelogenous leukemia.	(1+),neg.	-	-
HS	F/33	9 units blood during 3 previous days. Decreased platelets. Acute leukemia. No earlier record available.	-	23%	2+
NL	M/35	3 units blood 6 wks. ago following an accident and splenectomy. Platelets subsequently reached nearly 1,000,000/mm^3. No other history available.	3+,neg.	20%	2+
LS	F/28	Surgery 7 mos. ago required 12 units blood on 2 successive days and 8 units on 1 day, 1960.	neg,(neg.)	-	-

MM	M/51	During previous 6-12 mos. cardiac surgery twice, requiring over 10 units blood each time. 6 units transfused on another occasion.	neg.	8%	3+
FR	M/58	Surgery 9 mos. ago required 13 units blood; 8 mos. ago received 3 units packed cells in 2 days.	neg.	-	-
JD	M/53	Received during past 6-24 mos. (5 occasions) 12 units blood. Arteriosclerotic disease.	-	14%	+
RD	M/34	Cardiac surgery required 17 units blood 14 mos. ago.	-	40%	+
EC	F/55	Cardiac surgery 1964 required 10 units blood; received 2 units on 10th day post-op.	neg.	-	-
WM	M/65	Cardiac surgery 27 mos. ago required 13 units blood in 2 days.	-	38%	neg.
GB	F/48	Transfusion 2 units 1959, para ii 1959. Renal			
		insufficiency 1966.	neg.	21%	+
DM	M/68	Surgery 1959 required 6 units blood in 2 days. Cardiovascular disease, 1966.	-	35%	neg.
WB	M/75	In 1955 received 1 unit blood. Carcinoma 1966.	-	41%	neg.
HR	M/57	Received 1 unit blood 1947. On anticoagulant therapy 1966.	-	33%	neg.

Note: Results bracketed () were obtained when sera was incubated with platelets from blood freshly drawn from one individual.
Two results listed vertically for one patient indicate 2 blood samples withdrawn on 2 days.

Table V

Results Obtained on Non-Transfused Individuals

Patient	Sex/Age	Medical History	Results 1/12 Serum dilution test (C' free)	1/3 Serum dilution test (C')	1/15 Serum dilution test (C')
LF	F/60	Thrombocytopenia - secondary to infection or drugs. Decreased megakaryocytes in bone marrow. Platelet 5,000/mm^3. 6 units blood received in 1 wk. between serum #1 and serum #4.	2+ (2+),neg. - - 2+	- - - 11% -	- 2+ - 4+ -
MP	M/57	Chronic constipation, achlorhydria, hepatitis, arthritis, partial paralysis due to cerebrovascular accidents. Drugs regularly prescribed for joint pain include "ASA", "292's". Blood in stool (unconfirmed), vague history of gastric ulcer.	-	20%	3+
MD	F/34	Para ii (1959,1961), received talbutamide.	-	13%	3+
GS	F/16	Thrombocytopenia, receiving steroid therapy. Coombs test for platelet antibodies neg. History of easy bleeding and self-administration of TRC's (contains ASA).	neg. neg. neg. + neg.	- - - - 6%	- - - - 2+
MC	F/21	Hay fever, allergy to feathers and flowers, allergic rash recently successfully treated with cortisone ointment.	2+,4+,1+,(2+)	-	-
GG	M/47	G.I. bleeding, coronary artery disease, gout, mild diabetic (receiving Diabenese)	2+	-	-
DA	M/adult	Apparently healthy staff member. Infectious hepatitis 1948, no other history available.	(2+),1+,neg.	-	-

AM	F/46	Allergic to oat dust, renal hypertension	+,+	-	-
JK	M/66	Thrombocytopenia (platelets 40,000/mm^3). Allergy to sulfa drugs. Chronic intestinal infection, increased gamma globulins in blood.	neg.	7%	+
HC	M/52	Stroke 1966; myocardial infarct 1964. (On anticoagulant-Sintrom)	-	5%	+
DB	M/8	Thrombocytopenia, unknown origin, undergoing spontaneous remission.	neg. neg.	- -	neg. -
HE	M/11	Thrombocytopenia, leukopenia, splenomegally, no transfusions, C' consumption test "neg." for platelet antibodies.	neg.	-	-
RG	M/86	Thrombocytopenia, under remission after withdrawal of chlortripilon.	neg.	-	-
MS	F/54	Decreased platelets; para ii (1940), lymphosarcoma 1966, with bone marrow infiltration.	neg.	-	-

Note: Bracketed results () were obtained when test sera was incubated with platelets from blood freshly drawn from one individual.
Number of results listed vertically for one patient indicate the number of sera tested.

were obtained on sera of 18 individuals who had received transfusions for any reason. The list is compiled in order of increasing time lapse between the transfusion(s) and the platelet antibody test.

a. Discussion

Results of the 1/15 serum dilution test on 12 patients can be seen in the columns at the far right of Table IV, graded according to the system described in Section IV.C.2., i.e. 1+ to 4+ as the inhibition of 5HT uptake increased.

Among these 12, sera of 8 were found to have significant inhibition of 5HT uptake, indicating the presence of platelet antibodies. The sera of 3 who had received over 10 units of blood during the past year gave 3+ and 4+ results. Weaker positives were obtained on sera of 4 patients who had received between 3 and 9 units of blood in the 2 months preceding the date of the uptake study, or over 10 units between 6 and 24 months preceding that date. Patient WM, who received 13 units 27 months previous, was found to be negative for platelet antibodies, as were 3 patients who received less blood in the more distant past.

The 1/12 serum dilution system gave weaker positive results than did the 1/15 serum dilution method, and as well showed decreased sensitivity (see Section V.B.2.b.).

The hypothesis that many blood transfusions cause platelet antibody formation, has been borne out by this study. It may be that isologous antibodies are present in serum as soon as 3 days post-transfusion (patient HS) and as

long as 1-2 years after a large transfusion (patient RD). However, it should be determined in the case of multiple myeloma, for example, if, in fact, the anti-platelet-5HT-binding factor is the result of the infusion of isologous platelets, or is related to the primary disease process.

An interesting result is that for GB, who had, according to available records, received no transfusion since 1959. The possible significance of her pregnancies is outlined in Section V.B.3.

b. The 1/12 Serum Dilution Test (A Comparison)

The 1/12 serum dilution method was discontinued when, using the results obtained on transfused patients (Table IV), it compared unfavorably with the 1/15 serum dilution method in which complement (C') was the significant addition. This comparison was made in two ways.

Although 5 of 10 transfused patients tested by the 1/12 serum dilution method were found to have positive results, each of these had received at least some of their transfused blood within the past 2 months. Using the 1/15 serum dilution method, positive results were obtained on individuals who had not been transfused in the past 6-14 months. Further, one serum (patient MM), negative for antibodies when tested by the 1/12 serum dilution method, was 3+ when the 1/15 serum dilution method was used.

Since it was not likely that the difference in serum dilution (1/12 or 1/15) had any significant effect, the addition of C' to the test medium was probably responsible for the marked enhancement of sensitivity.

3. Results Obtained on Non-Transfused Patients

a. Discussion

In addition to tests on individuals who had received transfusions, tests were conducted on 34 non-transfused individuals. Of these, negative results were obtained on 12 healthy volunteers and for 8 patients with non-hematological disorders; i.e. abortion, chronic constipation, epilepsy, coronary artery disease, asthma, etc. The normal 5HT uptake values on these individuals tends to confirm the specificity of the test, although sera from a much larger series of miscellaneous disorders should be done.

Of the remaining 14 individuals tested, 10 (including 3 thrombocytopenics) had positive results. These, along with the data on four thrombocytopenic individuals who had negative results, are recorded in Table V.

Of the 7 thrombocytopenics, 4 were found to have negative sera. These included patients DB and RG whose conditions at the time of testing underwent spontaneous remission. The third, HE, age 11, was thrombocytopenic for no known reason; the last one, patient MS, had lymphosarcoma with bone marrow infiltration. Had it been possible to repeat these latter two tests, using the more sensitive 1/15 serum dilution method (containing C'), positive results might have resulted, since Cohen (29) lists both lymphosarcoma and ideopathic acute thrombocytopenia of childhood as primarily the result of increased peripheral destruction of platelets, rather than failure to produce platelets. Significant in the evaluation of the results here, Baldini (4) considers that many, if not

most, instances of increased peripheral destruction are due to immune processes.

Of the 7 thrombocytopenics, the sera of 3 were found to be positive. However, these (patients LF, GS, JK) as well as 5 of the remaining group of 7 non-thrombocytopenics listed in Table V, each have a history including one or a combination of the following factors which have been implicated in platelet disorders.

i. Infections

Baldini (30) notes that viral infections may precede thrombocytopenia and trigger both the defective production of platelets and peripheral destruction of platelets. He hypothesizes that an immune process is involved. For example, either the antigen is a virus-platelet unit, or the virus-antigen plus its antibody after union, attach to the platelet nonspecifically, altering it so that it is then destroyed.

ii. Drug Ingestion

Many drugs have been incriminated (in the literature) in one or more cases of thrombocytopenia. An immune process is the probable mode of action (4). Some of the drugs so listed and pertinent to the present discussion are Orinase (an oral hypoglycemic agent containing tolbutamide), phenacetin (contained in "292's"), sulfonamides, acetylsalicylic acid (ASA), and antihistaminics (13,28,30).

Theoretical mechanisms of platelet damage by drug have been outlined in detail in Section II.A.3. Briefly, these are (a) the platelet damage may result when a platelet protein becomes attached to the hapten (drug) to constitute an antigen.

The antibodies subsequently formed may have specificity not only for the hapten–protein combination but also for either part alone. A more widely accepted theory is that (b) an antibody may form in response to the presence in the blood, either free or attached to a plasma protein, of a drug or its metabolite. The resultant antigen-antibody complex may adsorb nonspecifically onto platelets, damaging, for example, 5HT uptake sites.

iii. A vague or documented complaint of easy bleeding or frequent g.i. bleeding.

iv. A history of allergy which may be significant in itself or because of the use of antihistaminics which may be implied. Allergic skin disorders (rash) have been recorded in the immediate history of acute thrombocytopenic purpura (4).

v. Pregnancy. Platelet isoantibodies may be found in maternal serum after one pregnancy (Section II.A.3). The survival time of these antibodies is unknown. Indeed, Morrison (27) describes a case in which a 55 year old woman (para ii, gravida iv, 25-30 years earlier) developed thrombocytopenia after her first transfusion (2 units AB and Rh compatible blood).

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Positive results obtained on the sera of two remaining individuals (DA and HC), cannot be explained, although it should be noted that complete medical histories are not available.

b. Conclusion

Since, with the possible exception of patient MD, the

histories in Table V do not include requisite conditions for the formation of isologous antibodies, it might be concluded that "autologous" antibodies are present in the positive serum. The other possibility is that products of an independent immune reaction are free in some test sera, and during incubation are able to adsorb nonspecifically to the platelets. In any case, each test sera which was found positive, contained factors, probably the result of an immune process, which are capable of inhibiting 5HT binding by normal platelets.

4. The 1/3 Serum Dilution Test

It was proposed that as a part of routine screening for platelet antibodies in serum, two serum dilutions be used in order to detect more accurately a wide range of possible antibody concentrations. Thus the 1/3 serum dilution system (Section IV.C.1.) was tried and the results obtained by this method can be seen in Tables IV and V, reported as a percentage of the 1/3-method saline control

In all, 19 tests using the 1/3 serum dilution method are reported. 5HT binding ranged from 1% to 41% of the saline control. It is hypothesized that this comparatively low range for results is an indication that the high concentration of serum is sub-optimal for platelet 5HT binding.

However, the results obtained by this method can be categorized by comparing them with results obtained by the 1/15 serum dilution system. The general ranges are: over 25%, 15-25%, and below 15% uptake by the 1/3 serum dilution system. These compare roughly with results in the 1/15 serum dilution

system graded: "normal", 1+ and 2+, 3+ and 4+, respectively. There are obvious exceptions, however (JK and HC, Table V), and whether these represent significant differences in the physiological effect of the antibody is not known.

The results of the protein assay for this test, after incubation of serum with platelets, are not nearly as constant as they are for the 1/15 serum dilution test. It is felt that this is another undesirable effect of the high concentration of serum (protein) in the incubation medium. It is possible that changes in serum protein, particularly after storage, may result in some protein precipitation during the incubation, which by centrifugation is added to the platelet button and hence is included in the value obtained by platelet protein assay. The false increase in the "platelet protein" value results in a decrease in the ratio "μg 5HT/mg platelet protein" and may therefore result in a "false positive" evaluation of antibody content in serum.

Of further interest when comparing the two methods is the fact that serum having a high antibody titer may inhibit less effectively 5HT binding in 1/3 dilution (about 25% uptake) than in 1/15 dilution (11% uptake). It is possible that at the higher concentration of antibody, a prozone inhibits effective antigen-antibody union, thus allowing the increased platelet-5HT binding. This observation eliminates the possibility of using the 1/3 serum dilution test to the exclusion of the 1/15 serum dilution test.

Further study, with low levels of antibody in serum is

needed to determine whether this 1/3 serum dilution method is a useful addition to the 1/15 serum dilution test in screening for platelet antibodies.

5. Comparison of Platelet 5HT Binding Tests with Others Available for the Detection of Platelet Antibodies

It is most likely that this simple method of screening for platelet antibodies by their inhibitory effect on platelet 5HT uptake is at the least as sensitive as any of the commonly used tests for platelet antibody detection.

a. Bridges (7) using an anti-platelet-5HT binding assay (similar to the 1/12 serum dilution method) tested sera from 4 patients who had received transfusions, and found evidence of antibody in each. When he tested the same sera by the method of Dausset (29), a procedure involving visible detection of agglutination of platelets, only 2 were found to be positive.

b. Serum on patient AK (Table IV) when tested for platelet antibodies by the Coombs' test, gave negative results. The 1/3, 1/15 and even the less sensitive 1/12 serum dilution methods each gave positive results on serum from this patient.

c. One of the traditional methods of detection of platelet antibody is to detect a clearing of a platelet suspension when antibodies cause cell lysing. In the present investigation, a clearing of the suspension being incubated with 5HT was sometimes noted, but only when a 4+ inhibition of 5HT uptake occurred.

Thus, the platelet-5HT binding method for the detection of platelet antibodies indeed compares favorably with three of the traditional methods of platelet antibody detection.

C. PLATELET 5HT BINDING TEST FOR DAMAGE TO PATIENT'S PLATELETS IN VIVO

1. Background

As ideopathic thrombocytopenia frequently occurs in the absence of evidence of a suspected autologous platelet antibody, it has been hypothesized that when the antibodies are produced, they are bound to the antigenic platelet sites rather than remaining free in detectable quantities in the blood. An alternative possible explanation is that the platelet destruction is secondary to an independent reaction involving a drug or allergen, free in blood or incidentally absorbed onto the platelet surface. Autologous platelet damage may also result by absorption of a theoretical complex which results from an isologous-platelet-antibody action. If either the "autologous platelet antibody" or "secondary damage" hypotheses were true, it is not surprising that results are negative, when test conditions are those designed for the detection of platelet antibodies per se.

In this study, positive results were obtained primarily on individuals whose history suggested the presence of isologous platelet antibodies (transfused individuals), but also on individuals (non-transfused) whose history indicated that platelet antibodies were not the cause of the anti-platelet-5HT-binding action. Since, in addition, positive results were obtained not only in instances of documented bleeding or thrombocytopenia,

the possibility arose that tests using autologous platelets might reflect more accurately the patients' symptoms. Thus the platelets as well as the serum of one individual were studied as follows.

2. Tests

Patient BA, has had many transfusions, 2 years earlier suffered fever-chill type transfusion reactions, was not thrombocytopenic (149,000/mm^3) but had uncontrolled bleeding which could indicate some type of platelet defect. Her serum when tested by the 1/15 serum dilution method for the presence of apparent antibodies, was found to be 3+ (30% of saline control). Next the patient's platelets freshly obtained and prepared, were incubated with labeled 5HT in the presence of saline using the conditions of a 1/15 dilution procedure. The uptake was found to be 0.58 μg 5HT/mg platelet protein. This represents a low uptake (about 50% of a comparable 1/15 saline control using pooled normal platelets). This lack of ability to bind normal quantities of 5HT would indeed indicate that there was in vivo alteration of the platelets. A 1/15 serum dilution test using the patient's platelets in the presence of her own serum showed a further decrease of 5HT binding to 0.16 μg 5HT/mg platelet protein, or 28% of the uptake of the patient's platelets in a saline control.

Therefore, not only did the patient's (autologous) platelets appear to be altered in vivo, but the serum factor further altered the uptake by both autologous and isologous platelets in vitro.

3. Discussion

In reviewing the above results, it is interesting that the same degree of platelet-5HT-binding inhibition occurred if patient serum was incubated with either (a) an isologous platelet pool, or (b) her own (autologous) platelets. This similar inhibition of uptake (a) 30%, and (b) 28%, may support a theory of nonspecific platelet damage (i.e. secondary to another reaction). As in the present case (patient BA), this damage may interfere with platelet function (hemostasis), but is not necessarily the cause of or coincident with thrombocytopenia.

It is anticipated that future platelet-5HT-binding studies should include saline controls on autologous platelets as well as normal platelets, and a test of the effect of patient serum on both. These studies might reveal the relationship between damage to platelets *in vivo* and the presence of (a) an anti-platelet-5HT-binding factor in serum, (b) thrombocytopenia and/or bleeding. The specificity of the factor could also be determined. Absorption studies may assist, although an absorption of antibody may be undetectable if after an antibody-platelet union there developed the hypothetical complex in serum which will contribute to its anti-platelet-5HT binding action. It is not known whether this complex would attach strongly to platelets, thus facilitating its subsequent removal from the serum (14).

VI. CONCLUSION

1. To test a proposed method of platelet tagging and estimation of survival of autologous platelets *in vivo*, volunteers were administered 5HT orally, for periods of 7-27 days, and platelet 5HT was assayed at appropriate intervals both during and following the administration of 5HT. In three successful trials, the platelet 5HT levels were increased to a level where it was possible to measure the disappearance rate and calculate the T1/2 or platelet half-life. Values from 3.5 to 5 days were obtained. These figures are compatible with estimates of platelet survival using other techniques. However, the number of days involved in loading as well as the failure in some instances to achieve elevated platelet 5HT levels after prolonged administration of 5HT, results in a procedure of little clinical value.

2. Subcutaneous injection of human platelets into rabbits has effectively stimulated *in vivo* production of an antibody or serum factor which inhibits the uptake of 5HT-C^{14} by normal human platelets. When an adjuvant, commonly used to facilitate a strong and persistent antibody formation, was also injected, there was more effective production of the anti-platelet-5HT-binding factor. The inhibition of platelet 5HT binding was effective until the dilution of sensitized rabbit serum in the incubation was over 1/100. This factor, further, can be absorbed out of rabbit serum by normal human platelets, thus

providing further evidence of antibody-like activity.

3. The sera of over 50 individuals has been studied for the presence of an anti-platelet-5HT-binding factor. The factor, identified by its effect on 5HT binding by normal human platelets, was not found in sera of normal individuals, but was, as expected, identified in the sera of those individuals who had within the past two years undergone extensive blood transfusion. This finding was compatible with the hypothesis that the lack of platelet-5HT-binding was the effect of the presence of isologous platelet antibodies resulting from transfusions with isologous platelets.

However, an anti-platelet-5HT-binding factor was also present in the serum of non-transfused individuals, either thrombocytopenic or non-thrombocytopenic, whose hospital medical records indicated (a) frequent administration of drugs (e.g. acetylsalicylic acid and oral hypoglycemic agents), or (b) the presence of one or more allergies. The fact that the anti-binding factor was detected in this diverse group of individuals implies that its origin may be one of a range of conditions which have by various authors been implicated in cases of "auto-immune" or "idiopathic" thrombocytopenia.

4. Because of the lack of correlation between the presence of anti-platelet-5HT-binding factor and the occurrence of thrombocytopenia and/or bleeding in non-transfused individuals, the question arose of the need to study not patient serum but patient platelets. One study was made. The patient, a multi-transfused female, was not thrombocytopenic but suffered from

uncontrolled bleeding. Data obtained indeed indicated an *in vivo* alteration of the platelets as well as the presence in serum of an anti-platelet-5HT-binding factor which was equally damaging to autologous or isologous platelets *in vitro*.

5. Therefore, while the anti-platelet-5HT-binding test cannot be said to be a means of detection of platelet antibodies per se, there is evidence that it could be a useful indicator of the types of alteration which may lead to peripheral destruction of platelets.

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